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ISOLATION AND CHARACTERIZATION OF CHYMOTRYPSINOGEN B

by

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ABSTRACT

Chymotrypsinogen-B, the enzymically inactive precursor of the proteolytic enzyme chymotrypsin-B, has been purified from a sulfuric acid extract of beef pancreas glands and characterized with regard to molecular weight, sedimentation behavior as a function of pH and protein concentration, viscosity, and isoelectric point. The specificities of chymotrypsin-A_4 and chymotrypsin-B have been compared using glucagon as substrate.

In order to achieve the best preparation of chymotrypsinogen-B as judged by potential chymotryptic activity and homogeneity, the merits of various available techniques were tested. It was found that purification of the zymogen by chromatography on diethylaminoethyl cellulose columns, pH 8.0, was not a useful procedure. At this pH, considerable proteolysis took place, resulting in a product of only 70 per cent homogeneity, as evaluated by the stoichiometric reaction between cinnamoyl imidazole and chymotrypsin-B. Stepwise elution of the protein from carboxymethyl cellulose at pH 4.6 was found to be dependent on the ionic strength of the buffer and the capacity of the resin. This procedure resulted in a product estimated to be 80 per cent homogeneous. A method involving gradient elution of the zymogen from carboxymethyl cellulose at pH 4.47 was developed and shown to yield 95 per cent homogeneous chymotrypsinogen-B. One per cent by weight of deoxyribonuclease was present in the final product. It was also demonstrated that 100 per cent pure chymotrypsin-B should exhibit a zero order rate constant of hydrolysis of N-acetyl-L-tyrosine-ethyl-ester equal to 3.36.

Preparations of 85 per cent purity or better gave rise to a single symmetrical peak in the ultracentrifuge and electrophoresis apparatus when tested in buffers of 0.1 ionic strength over the pH range 3.0 to 7.5.

The molecular weight of chymotrypsinogen-B has been determined by light scattering, approach to sedimentation equilibrium, and sedimentation-viscosity. The values obtained by these methods are, respectively, 25,000 $\pm$400, 24,700 $\pm$900, and 24,000 $\pm$2,000. They tend to converge towards 24,500 as the most probable molecular weight of chymotrypsinogen-B. Sedimentation-velocity runs and molecular weight determinations showed that chymotrypsinogen-B existed as a monomer in solutions with pH values equal to or lower than 4, but aggregated with increasing protein concentration and pH. The pH of zero mobility in 0.1 M acetate buffer was determined as 5.2.

Digests of glucagon with chymotrypsin-A_4 and chymotrypsin-B have been compared using two-dimensional separation of the resulting peptides by high-voltage electrophoresis and paper chromatography as well as analysis of the amino-terminal amino acids by the fluorodinitrobenzene assay. Evidence is presented indicating that chymotrypsin-B is less specific than chymotrypsin-A_4. Under the reported conditions of the digestion of glucagon, it was found that chymotrypsin-B catalyzes the hydrolysis of peptide bonds involving the carboxyl group of leucine in addition to those split by chymotrypsin-A_4. No evidence was found supporting a possible contamination of the enzyme preparation with a leucine esteroproteolytic enzyme.

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ABBREVIATIONS

CHTG	-	Chymotrypsinogen
CHT	-	Chymotrypsin
DNase	-	Deoxyribonuclease
C-terminal	-	Carboxyl-terminal
N-terminal	-	Amino terminal
STI	-	Soybean trypsin inhibitor
DEAE-C	-	Diethylaminoethyl-cellulose
CM-C	-	Carboxymethyl-cellulose
Tris	-	Tris (hydroxymethylamino) methane
DFP	-	Diisopropylfluorophosphate
ATEE	-	N-acetyl-**L**-tyrosine ethyl ester
ATryEE	-	N-acetyl-**L**-tryptophane ethyl ester
BLEE	-	N-benzoyl-**L**-leucine ethyl ester

I. INTRODUCTION

The classical work of Northrop and Kunitz demonstrated the existence of high potential protease activity in acid extracts of pancreas glands. Two zymogens, trypsinogen and chymotrypsinogen-A* (CHTG-A), were isolated and partially characterized. Since that time, a number of other zymogens and their corresponding enzymes have also been demonstrated in extracts and the excretory juice of beef pancreas glands. Of these, trypsinogen, procarboxypeptidase-A, CHTG-A, and chymotrypsinogen-B (CHTG-B) are present in the highest concentrations (2), although the presence of procarboxypeptidase-B and carboxypeptidase-B has also been demonstrated (2). In addition to the proteolytic enzymes, ribonuclease (3), and deoxyribonuclease (4) have been isolated and characterized, but only low levels of amylolytic and lipolytic activity were found in beef pancreas glands (2).

In the last decade, CHTG-A and its activated form have received ample attention, stimulated in part by the relative ease with which the zymogen as well as the enzyme could be prepared in large quantities from fresh beef pancreas glands (4).

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*Following the proposal of Rovery and Desnuelle (1), chymotrypsinogen-α will be termed chymotrypsinogen-A; π, δ, and α-chymotrypsin will be called chymotrypsin-A_1, A_2, and A_4, respectively.

It is therefore not surprising that an impressive body of knowledge has accumulated over the years. It is beyond the scope of this chapter to review every study that has been made on CHTG-A. However, since part of this work was designed as a comparison between the two pancreatic proteins exhibiting chymotryptic specificity, some of the general properties of CHTG-A will be mentioned in the Introduction, and other pertinent references will appear, where necessary, throughout the thesis.

In 1930, Kunitz described the crystallization of CHTG-A obtained from sulfuric acid extracts of beef pancreas glands (5). Although considered for many years as one of the most homogeneous proteins, some preparations, even after several crystallizations, may still be contaminated by "neochymotrypsinogens",* as demonstrated by end group analysis (6); or other impurities, as shown by chromatography on IRC-50 ion exchange resin (7) or countercurrent electrophoresis (8).

The chemical events accompanying the activation of the zymogen to the enzyme have been studied in great detail by two groups of investigators (6,9). Their results indicate that the splitting of a single peptide bond by trypsin during the early stage of rapid activation is associated with the appearance of full enzymic activity. Chymotrypsin-A_1, the very unstable intermediate, then autolyzes the second bond of one of its carboxyl-terminal (C-terminal) sequences, giving rise to chymotrypsin-A_2 and the dipeptide seryl-arginine.

*Term used as suggested by Rovery et al. (6).

During slow activation, CHTG-A is slowly attacked by chymotrypsin-A_4, and through the cleavage of two bonds, located in another region of the molecule, a second dipeptide, threonyl-asparagine, is liberated. In the absence of trypsin, this results in the formation of neochymotrypsinogens without activation, whereas in its presence neochymotrypsinogens are transformed into chymotrypsin-A_4 (CHT-A_4), which can also arise from chymotrypsin-A_2 by autolysis.

In all, four peptide bonds are cleaved during the conversion of CHTG-A to chymotrypsin-A_4 and three chains, held together by disulfide bridges, are formed. Meedom (10) achieved the purification of chain A, which contains 13 amino acid residues and represents the amino-terminal (N-terminal) of the zymogen, and almost completely established its structure by the classic techniques of partial degradation (11). Chain C, constituting the C-terminal end of CHTG-A, has recently been purified by chromatography on Sephadex columns (1). Nothing is known thus far about the purification of chain B. Studies on the complete amino acid sequence of CHTG-A have progressed far enough to ensure completion within a few years (12).

Much time has been devoted to studies on the active site of the enzyme as well as on its mechanism of action. Detailed kinetic studies of chymotrypsin catalyzed reactions (13), as well as labelling of the active site with diisopropylfluorophosphate (DFP) or paranitrophenylacetate (14,15) gave valuable information on the amino acid(s) presumably involved in catalytic

action. Considerable evidence supports the view that imidazole functions as a general basic or nucleophilic catalyst in the hydrolysis of model substrates by CHT-A_4 (16,17).

The molecular weight of CHTG-A has been well established by physico-chemical as well as by chemical methods. Light scattering (18,19), sedimentation diffusion (19), X-ray diffraction (20), and osmotic pressure measurements (7) suggest 25,000 as the most probable molecular weight. These data are in close agreement with the value found by amino acid analysis of the zymogen (19).

Compared to the extensive studies on CHTG-A in many laboratories over the years, the properties of CHTG-B and its active enzymes have received little attention. Undoubtedly, this lack of interest has arisen from the lack of satisfactory methods for the isolation and purification of the zymogen in good yield. Furthermore, it was not appreciated until recently (2) that the B zymogen comprised 16 per cent of the proteins of the pancreatic juice of cattle.

The isolation and crystallization of an unknown protein from beef pancreas glands was reported by Laskowski in 1946 (21). Originally considered to be a deoxyribonuclease, the protein was soon demonstrated to possess proteolytic activity (22,23,24) and the nucleolytic activity to be due to contamination with deoxyribonuclease. Since the zymogen could be activated by trypsin and enterokinase, and since it also formed a dissociable complex with soy bean trypsin inhibitor (STI), and clotted milk, it was

suspected to be related to CHTG-A. This was confirmed by Fruton who showed the enzyme to have a specificity, similar to chymotrypsin-A_4, towards synthetic peptide derivatives of phenylalanine and tyrosine (25). Brown et al. (26), who achieved the crystallization of the active protein, suggested the names chymotrypsinogen-B and chymotrypsin-B (CHT-B) for the zymogen and its activated form in 1948.

A. Preparation and purity of CHTG-B

Laskowski and co-workers (27) originally prepared CHTG-B by ammonium sulfate fractionations of the sulfuric acid extract of fresh beef pancreas glands (5). In this procedure, the zymogen was first precipitated from the extract with ammonium sulfate between 0.2 and 0.4 saturation. The precipitate was subjected to two further fractionations between 0.2 and 0.4 saturation; first from water and then from 0.2 M sodium acetate buffer at pH 4.0. The resulting precipitate was dissolved in a minimum amount of water at pH 4.0 and crystallized by dialysis against 0.01 M sodium acetate buffer, pH 5.5. Several recrystallizations increased the purity of the preparation significantly. Chymotrypsin-B could be crystallized by almost the same method after activation of the zymogen with trypsin at pH 7.8 for four days (molar ratio of trypsin to chymotrypsinogen: 1 to 2,000).

Since CHTG-B prepared by the above method always contained up to three per cent of the activated form, Laskowski and Kassel undertook chromatographic studies with both proteins (28). Crystalline preparations of CHTG-B were subjected to column

chromatography on carboxymethyl cellulose (CMC) columns previously equilibrated with 0.1 M sodium phosphate buffer, pH 4.5. It was shown that this technique differentiated between the active and non-activated forms but did not resolve native CHTG-B and neochymotrypsinogens. The latter conclusion arose from studies of the C-terminal amino acids of the zymogen preparations, which revealed significant quantities of tyrosine, phenylalanine, valine, and leucine. It was also possible to demonstrate the existence of more than one form of active chymotrypsin-B by chromatography on diethylaminoethyl cellulose (DEAE-cellulose). One of the several chymotrypsins could be crystallized and shown to be of a different form than any previously observed.

These observations led Kassel and Laskowski to modify their method for the preparation of CHTG-B by the introduction of 0.01 M DFP* into solutions with a pH greater than 4.0 (29). It was thereby possible to reduce the amount of C-terminal tyrosine to 5-7 per cent and the free activity to 0.2 per cent of a standard preparation.

Laskowski's laboratory determined the activity of CHT-B by the method of Kunitz (30) with casein as substrate. Hydrolytic potency was expressed in per cent of the standard chymotrypsin-B. The activity of the standard chymotrypsin was the average obtained from a large number of chymotrypsin preparations (31). The activity of the enzyme was read from a standard curve which related the increase of the optical density of a 1 per cent casein solution during digestion with μg of CHT-B used.

*Diisopropylfluorophosphate

Rovery et al. reported a somewhat different method for the preparation of CHTG-B in 1960 (32). After an initial fractionation of the sulfuric acid extract with ammonium sulfate between 0.2 and 0.4 saturation, the precipitate is dissolved in 0.001 M HCl and dialyzed against the same dilute acid and freeze-dried. After solution in 0.05 M citrate buffer, pH 4.2, the protein is applied to a carboxymethylcellulose (CM-cellulose) column previously equilibrated with the same buffer. After the appearance of a small inactive peak, CHTG-B can be eluted with 0.05 M citrate buffer, pH 4.6. Rechromatography on the same column yields a completely inactive preparation with high potential activity.

Chymotrypsinogen-B, converted to the enzyme by the rapid activation procedure (33), shows maximal activity after 30 minutes, as recently reported by Rovery et al. (34). The highest activity of CHT-B when measured against N-acetyltyrosinethylester (ATEE) as substrate and expressed as the zero order rate constant of hydrolysis (k' = meq of substrate hydrolyzed per minute per mg of enzyme nitrogen) was 2.6 (based on $E^{1\%}_{1\,cm}$ = 18.0 at 280 mμ (27)).

Both laboratories claim to have a reasonably pure preparation. Rovery et al. base their conclusions on equilibrium chromatography of the purified protein on DEAE-cellulose and the observation that the activity curve of individual column fractions follows the concentration curve very closely (32). The finding of only one N-terminal amino acid also supports this conclusion.

Laskowski's group showed that the protein was electrophoretically homogeneous at three different pH values (35) and could find no evidence of heterogeneity in the ultracentrifuge (36). Solubility studies (35) revealed a small amount of impurity present in their preparations. The presence of small amounts of C-terminal tyrosine indicated contamination with neochymotrypsinogens (29).

B. Amino acid composition and terminal amino acids of CHTG-B

The amino acid composition has been investigated by two laboratories. Kassel and Laskowski found that it contained 219 residues, based on a molecular weight of 24,000 (29). The minimum molecular weight was calculated to be 23,086. It was shown that CHTG-B differs considerably from CHTG-A. The main difference lies in the number of amide groups which explains the difference in the isoelectric points of the two proteins (35).

Rovery and co-workers found 185 amino acids and calculated a minimum molecular weight of 21,215 $\pm$340 from their data (34). However, their analyses do not include tryptophane, half-cystine, nor amide groups.

If one corrects Laskowski's values to a molecular weight of 21,215, the two analyses agree fairly well, with the exception of two amino acids, namely, glutamic acid and methionine. It has been established, however, that Rovery's preparation is only about 80 per cent pure (see Results of Purification).

Good agreement exists between the two laboratories that the N-terminal amino acid is a half-cystine residue (32,37). In a more recent communication, Kassel and Laskowski reported the determination of the last three amino acids of the amino terminal to be NH_2-Cys-Gly-Val- (38), the same as in CHTG-A (11).

It seems difficult at the moment to name the C-terminal amino acid with certainty. Rovery et al. claim to have found leucine in molar quantities by the hydrazinolysis method (34). Carboxypeptidase-A, in the absence of urea, liberated only small quantities of leucine, valine, and alanine, as well as traces of asparagine. In the presence of urea (concentration not stated), numerous amino acids were liberated by carboxypeptidase-A, the identity of which has not as yet been revealed.

Gladner and Neurath also studied the C-terminal groups of CHTG-B on a preparation supplied by Dr. Laskowski (39). Carboxypeptidase-A liberated 0.2 moles of leucine and 0.6 moles of tyrosine from the zymogen, and 0.5 moles of leucine as well as 1.0 moles of tyrosine from the diisopropylphosphate derivative of the free enzyme. Their conclusion was that the enzyme had not been sufficiently pure to warrant end group analysis.

Kassel and Laskowski recently reported the C-terminal sequence of CHTG-B and CHTG-A (38). Their data indicated that the arrangement of the last six amino acids in both proteins was the same and that the zymogens differed with respect to the seventh amino acid only. The sequence identified was:

-Val-Thr-Ala-Leu-Ala-AspNH_2. The seventh amino acid seems to be glutamic acid in the case of CHTG-B and either valine or asparagine in the case of CHTG-A.

C. Physico-chemical properties of CHTG-B and CHT-B

Investigations of the sedimentation behaviour of CHTG-A and B as well as CHT-A_4 and B by Smith et al. (36) revealed a high degree of homogeneity of the proteins. The sedimentation constant, $s^{\circ}_{20,w}$, was found to lie in the range of 2.50 to 2.65 S with the sedimentation constants of the enzymes being somewhat higher than those of the zymogens. Both zymogens showed very little concentration dependence of $s_{20,w}$ at pH 3.88, indicating highly symmetrical molecules and the absence of association. The numerical values of $s_{20,w}$ for the respective enzymes were highly concentration dependent and pH sensitive. While dimerization could be demonstrated with both, the pH dependence was found to be quite different for the two enzymes. CHT-B did not dimerize at pH 4.0, whereas CHT-A_4 exhibited association within the pH range 2.8 to 5.4. The diffusion constants of the proteins showed no significant differences and the values for the frictional coefficients confirmed the symmetrical nature of the proteins. Assuming a value of 0.73 for the partial specific volume of each protein, the molecular weights could be calculated and were found to fall between 22,000 and 24,000.

Values for the isoelectric point of CHTG-B and CHT-B have also been evaluated. Zero mobility in buffers of 0.1 ionic

strength was found at pH 5.2 for the zymogen and 4.7 for the enzyme (35).

The following table lists all the physico-chemical parameters which were known at the beginning of this study.

	CHTG-B	CHT-B	Reference
Isoelectric point	5.2	4.7	(35)
	5.1		(2)
$s^{\circ}_{20,w}$	2.49 S	2.65 S	(36)
	2.53 S		(2)
Diffusion constant	10.3×10^{-7} cm^2/sec	9.9×10^{-7} cm^2/sec	(36)
f/f_o	1.11	1.13	(36)
M.Wt.	21,600	23,600	(36)

Since then, the molecular weight of CHTG-B has been evaluated from amino acid analyses by Kassel and Laskowski (29) as well as by Rovery and Desnuelle (35). Their findings indicated minimum molecular weights of 23,086 and 21,215 $\pm$340, respectively.

D. Specificity and enzymatic properties of CHT-B

Since Fruton (25) characterized protein B, very little work has been done on the specificity of CHT-B. Keller, Cohen and Neurath used several synthetic substrates to characterize the proteins of pancreatic juice, and found that CHT-B hydrolyzed ATEE and N-acetyl-L-tryptophane-ethyl-ester (ATryEE), the latter at a much slower rate. Trace activity exhibited

towards benzoylglycyl-L-lysine was believed to be due to contamination with carboxypeptidase-B (2). Rovery et al. corroborated their finding and in addition showed that the decrease in activity of CHT-B towards ATryEE in 30 per cent methanol was much greater than for CHT-A_4 (34).

Enzymically CHT-B was found to be less efficient than CHT-A_4 (24,40,41). Ambrose and Laskowski demonstrated that the rate of digestion of casein, denatured egg albumin, edestin, denatured hemoglobin, and chymotrypsinogen-A proceeded more slowly with CHT-B than CHT-A_4 (40). Wu and Laskowski could show that CHT-B required calcium in the reaction mixture to enhance the activity and stabilize the enzyme as was previously shown to be the case for CHT-A_4 by Green et al. (42). In a system containing 0.1 M $CaCl_2$ and phenylalanine ethyl ester as the substrate, CHT-B was found to be twice as active at 30°C. and pH 6.5 as CHT-A_4.

It is clear that our understanding of the structure and enzymic activity of CHTG-B is at a low level in comparison to that of the CHTG-A system. Disagreement on the C-terminal amino acid(s) of the zymogen seems to arise from an unsatisfactory method of preparation. This in turn prevents studies from being carried out on the processes leading from the enzymically inactive protein to the enzyme. Very little attention has been devoted to the specificity of CHT-B, and it appears to the author that only a rough estimate of the molecular weight of CHTG-B is available at the present time. This

lack of knowledge prompted the study described in this thesis.

The objectives of the present study may be summarized as follows: (i) To establish a method of preparation of CHTG-B which would yield as homogeneous a protein as possible. Since it was clear from the beginning that ammonium sulfate fractionation and crystallization were inferior to column chromatography on CM-cellulose, the main efforts were directed to the latter approach. Nevertheless, chromatography on DEAE-cellulose columns and crystallization had to be examined also to establish their place among the existing procedures. (ii) To evaluate the molecular weight of CHTG-B by the Archibald method (approach to sedimentation equilibrium), light scattering, and by the relationship existing between the molecular weight of a macromolecule and its rate of sedimentation and intrinsic viscosity (43). (iii) To make a comparative study of the specificities of CHT-A_4 and CHT-B towards the polypeptide hormone glucagon, synthetic amino acid ester, and peptides. Since much time has been and still is devoted to the elucidation of primary structures of proteins, it was of interest to evaluate the usefulness of CHT-B in such studies. Glucagon was chosen as a substrate for two reasons: first, its primary structure has been elucidated by Bromer, Sinn and Behrens (44) using subtilisin, chymotrypsin-A_4, and trypsin; second, it contains a variety of amino acids and therefore resembles a protein molecule,

the natural substrate for proteolytic enzymes. In addition, it may be obtained commercially in a homogeneous form.

II. PREPARATION AND PURIFICATION OF CHYMOTRYPSINOGEN-B

The initial objective of this research was to find a method of preparation for the zymogen which would yield a highly homogeneous and potentially active protein. The procedures available when this work was begun may be summarized as follows: firstly, the isolation of CHTG-B by ammonium sulfate fractionation and crystallization according to the method of Laskowski *et al.* (27); secondly, the separation of freeze-dried pancreatic extract on diethylamino ethyl cellulose, pH 8.0, in accordance with the procedure of Keller, Cohen and Neurath (2); and thirdly, the stepwise elution of CHTG-B from carboxymethyl cellulose columns, pH 4.6, as described by Rovery and Desnuelle (32).

Since it was not known which approach would yield the best preparation with regard to potential activity and homogeneity, each method was tested. Experiments done in the early stages of this work indicated that the procedure devised by Laskowski (his chromatographic studies on CM-cellulose columns were published considerably later) did not yield a satisfactory product, involving as it did, fractionations close to neutrality. This caused the formation of considerable amounts of free proteolytic activity which made crystallization of the zymogen difficult. On several occasions, significant

reduction in the optical density at 280 mμ during dialysis indicated the occurrence of autolysis during the crystallization procedure.

In the following sections, investigations of the chromatographic behaviour of CHTG-B on DEAE- and CM-cellulose will be described. The merits of various methods of preparation will be discussed in terms of the homogeneity of the final product and a new procedure for the preparation and purification of the zymogen will be given.

A. Methods

1. Preparation of Protein Powder 1, 2, and 3 (PP 1, 2, and 3)

Ten to thirty beef pancreas glands were collected in a meat packing plant as soon as possible after slaughter. Fat and connective tissue were removed immediately and the cleaned glands were cut into small pieces before immersion in ice-cold 0.25 N sulfuric acid (three liters for ten glands). The suspension was then transferred to the cold room (4°C±1°) where all subsequent operations were carried out. The glands were ground in an electric meat grinder and resuspended in the acid, an additional liter of sulfuric acid being added for every ten glands. The volume of dilute sulfuric acid was found to be critical since too thick a suspension of precipitate after addition of ammonium sulfate made centrifugation difficult. After standing in the cold room for 24 hours, the suspension was strained with 4 layers of cheese cloth. The

residue was resuspended in dilute sulfuric acid (3 liters per 10 glands) and immediately filtered through cheese cloth. Reagent grade ammonium sulfate (114 g per liter) was added to the combined filtrates to bring to 0.2 saturation. After 30 minutes of stirring the precipitate was collected by centrifugation at 2,000 rpm for 30 minutes. The supernatant was brought to 0.4 saturation by the addition of 121 g of ammonium sulfate to every liter of the enzyme solution. After 30 minutes of vigorous stirring, the precipitate containing CHTG-B was collected by centrifugation at 2,000 rpm for 30 minutes. It was generally dissolved in a minimum volume of 0.005 M HCl and dialyzed against several changes of 0.001 M HCl for 24 hours. Any insoluble material was removed by centrifugation at 9,000 rpm for 60 minutes in a Servall high-speed centrifuge. After lyophylization, 12 g of protein powder 1 (PP 1) were reproducibly obtained from 10 glands.

In the method of preparation which was finally adopted by this laboratory, an additional series of fractionations with ammonium sulfate was introduced. The supernatant from the high-speed centrifugation was diluted to a 1.75 per cent solution ($E^{1\%}_{1\ cm}$ = 18.7 at 280 mμ, see Results) and adjusted to pH 3.0. A saturated solution of ammonium sulfate, brought to pH 3.0 by the addition of concentrated HCl, was then added slowly to obtain 0.25 saturation (25 ml for every 75 ml of enzyme solution). The resulting precipitate was collected by centrifugation (30 minutes at 2,500 rpm) and discarded. The

supernatant was brought to 0.30 saturation with ammonium sulfate (7 ml per every 100 ml of enzyme solution) and centrifuged. The precipitate was dissolved in a small volume of 0.005 M HCl, dialyzed against 0.001 M HCl, and freeze-dried to yield protein powder 2 (PP 2). To every 100 ml of the supernatant, 17 ml of saturated ammonium sulfate solution were added to attain 0.40 saturation. The suspension was centrifuged as above and the precipitate was processed as before to yield protein powder 3 (PP 3). Since PP 2 had considerable potential chymotryptic activity ($k'_{pot.} = 0.95$), it was saved and fractionated as above whenever enough material had accumulated.

2. Chromatography on DEAE-cellulose

The procedure worked out by Keller, Cohen and Neurath for the separation of the proteins of pancreatic juice (2) was adopted. DEAE-cellulose (Schleicher and Schuell Company, 0.9 meq per g) was purified according to Peterson and Sober (45). The resin was equilibrated with 0.005 M phosphate buffer, pH 8.0, and packed into a column, the size of which depended on the purpose of the experiment. For analytical runs, the dimensions were 1 cm x 60 cm; for preparative runs, 5 cm x 60 cm. One hundred mg of PP 1 and 2.5 mg of STI (5 g and 100 mg, respectively in case of large columns) were dissolved in 5 ml (250 ml) of 0.005 M phosphate buffer, pH 8.0, containing 0.001 M diisopropylfluorophosphate (DFP). The pH of the suspension was adjusted to pH 8.0, followed by centrifugation in a Spinco Model L centrifuge at 40,000 rpm for 30 minutes. The optical

density of the supernatant was determined at 280 mμ to allow the estimation of recoveries. After application of the protein solution to the column, a continuous flow of sodium phosphate buffer (0.005 M, pH 8, 0.0001 M DFP) was begun* and suitable fractions were collected in an automatic fraction collector. The optical density of each fraction was read at 280 mμ. It was shown by Keller, Cohen and Neurath that trypsinogen, ribonuclease, and chymotrypsinogen-A were not adsorbed on DEAE-cellulose at this pH and emerged as a large protein peak, i.e. the break-through peak, immediately after displacement of the hold up volume. The same was true for the experiments reported herein. When the material in the break-through peak had been collected and the absorption at 280 mμ had returned to a base line value, gradient elution was begun. A linear gradient from 0.005 M to 0.4 M sodium phosphate buffer, pH 8.0, containing 0.0005 M DFP, was established and a single peak was eluted, representing chymotrypsinogen-B. Five fractions along the eluted peak were collected from a preparative column, brought to pH 2.5 by the addition of 5 N HCl, dialyzed against 0.005 M HCl for 24 hours, and freeze-dried (CHTG-B - DEAE). The activity of each fraction was determined (see Appendix).

3. Chromatography on CM-cellulose

a. CM-cellulose (Bio Rad Laboratories, Inc., 0.9,

*Flow rate 0.4 ml per min. per square centimeter of column surface.

0.67, 0.59, and 0.50 meq per g) was purified according to Peterson and Sober (45). Protein PP 1 was used as starting material for analytical as well as preparative columns. The resin was equilibrated with 0.05 M sodium citrate buffer, pH 4.2, and packed into a column of 30 to 40 cm. Protein powder 1 (100 mg for analytical, 5 g for preparative columns) was dissolved in the same buffer, containing 0.001 M DFP. The solution was centrifuged at 40,000 rpm for 30 minutes prior to application to the column. The protein was washed into the column and 0.05 M sodium citrate buffer, pH 4.2, 0.001 M DFP, was passed through the column at a rate of 0.5 ml per minute per square cm of column surface. After a small break-through peak, the molarity of the buffer was changed to 0.05, 0.06, 0.07, or 0.08 M sodium citrate, pH 4.6, containing 0.005 M DFP, depending on the capacity of the resin. Fractions from different parts of the peak were pooled, brought to pH 2.5 by the addition of HCl, dialyzed, and freeze-dried (CHTG-B - CMC). The activity of each fraction was determined (see Appendix).

b. CM-cellulose (Bio Rad Laboratories, Inc., approximately 0.7 meq per g) was prepared as above and equilibrated with 0.03 M sodium citrate buffer, pH 4.47 in the cold room. It was packed into a 3 cm x 50 cm (for analytical purposes) or 6.5 cm x 50 cm (preparative) column. Protein powder 1 and PP 3 were used as the starting material. For analytical runs the linear gradient consisted of one liter of 0.03 M citrate buffer

and one liter of 0.15 M citrate buffer, pH 4.47. A 2 per cent solution of PP 1 or PP 3 in 0.03 M sodium citrate, pH 4.47, containing 0.005M DFP, was applied to the column and washed in with a few milliliters of the same buffer. Four hundred mg were charged onto the small column and 2 g onto the large one. Gradient elution was begun immediately and the flow rate was adjusted to 0.3 ml per minute per square centimeter of column surface. Eighteen ml fractions were collected in an automatic fraction collector and the absorbancy of each tube was read at 280 mμ. Various fractions were tested for free chymotryptic and potential chymotryptic and tryptic activity in the pH-stat. To determine the potential activities, the pH of one ml aliquots was adjusted to 8.0 by the addition of tris (hydroxymethylamino) methane (Tris) buffer (1 M) and suitable volumes of a 0.5 per cent solution of trypsin were added. After incubation overnight at 0°C. the esterolytic activity was estimated (see Appendix). Deoxyribonucleolytic activity was determined after dialysis of various fractions against 0.1 M sodium acetate buffer, pH 5.0, as outlined in the Appendix. For preparative purposes, all fractions with an absorbancy greater than 1.0 were pooled, and brought to pH 2.5. While stirring, a saturated solution of ammonium sulfate, pH 3.0, was added to yield 0.5 saturation. After stirring for 30 minutes, the precipitate was collected by centrifugation at 9,000 rpm for one hour. The collected precipitate was dissolved in 0.005 M HCl, dialyzed exhaustively against several changes of 0.001 M HCl, the solution clarified

by filtration through a millipore filter and freeze-dried (CHTG-B - G).

4. Determination of $E^{1\%}_{1cm}$ at 280 mμ, moisture and nitrogen content

Fifty mg of CHTG-B - G was equilibrated with laboratory air for 24 hours and divided into three portions. Fifteen mg was dissolved in 25 ml of 0.001 M HCl and the absorbancy was determined at 280 mμ. Another 15 mg was dried to constant weight in a vacuum oven over P_2O_5 at 105°±2°C. The remaining 20 mg was used for nitrogen analysis according to the method of Chibnall, Rees and Williams (46). The extinction coefficient ($E^{1\%}_{1cm}$ at 280 mμ) and nitrogen content were subsequently corrected for moisture.

5. Crystallization of CHTG-B - CMC

A 5 per cent solution of the protein in 0.0001 M HCl, containing 0.005 M DFP, was dialyzed against several changes of 0.01 M acetate buffer, pH 5.5, for 48 hours. The crystals were collected by centrifugation and the procedure was repeated. After one recrystallization, the crystals were dissolved in 0.005 M HCl, dialyzed against 0.001 M HCl for 24 hours, and freeze-dried (CHTG-B - C).

6. Ammonium sulfate fractionation of CHTG-B - CMC

Zymogen preparations with high potential chymotryptic activity were also prepared by ammonium sulfate fractionation of a 2 per cent solution of the protein obtained from carboxymethyl cellulose columns. The partial saturations were carried out

in 0.001 M HCl with a saturated solution of ammonium sulfate, pH 3.0. The precipitate, collected between 0.28 and 0.32 saturation, was dissolved in dilute acid, dialyzed, and freeze-dried as above (CHTG-B - ASF).

7. Analysis for C-terminal amino acids by carboxypeptidase-A

Worthington carboxypeptidase-A was used exclusively. The method employed was similar to that described by Gladner and Neurath (47). Quantities of CHTG-B - C, ASF, G equivalent to 1 μmole were digested with carboxypeptidase (0.08 μmoles) in the presence of 0.0025 M DFP for one hour at room temperature in 2 ml of 0.05 M phosphate buffer, pH 8.0. The reaction was stopped by adding aliquots of the digestion mixture to 100 mg of nalcite resin (hydrogen form) which then adsorbed the free amino acids leaving the protein in solution. The resin was washed with demineralized water, the amino acids eluted with 6 N ammonium hydroxide, and the eluate evaporated. The residue was dissolved in a small amount of demineralized water and subjected to high voltage electrophoresis and chromatography in the system butanol-butyl acetate-acetic acid-water, 19:1:5:25. This procedure is described in Section IV, together with the method for the quantitation of the amino acids.

8. Determination of the enzymic normality with cinnamoylimidazole

In a recent communication, Schonbaum et al. (48) described a spectrophotometric determination of the operational normality

of solutions of CHT-A_4. In brief, this method is based on the stoichiometric reaction of cinnamoyl-imidazole with the active site of CHT-A_4. Since it appears from other data that only one site is available for rapid acylation on the enzyme molecule, the operational normality determined by this assay, i.e. the number of active sites per molecule, can be translated into enzyme molarity. Thus this reaction provides a simple assay for the purity of a solution of CHT-A_4 provided its molecular weight and extinction coefficient are known with accuracy. In preliminary studies in this laboratory, it was found that CHT-B behaved similarly when tested with cinnamoyl imidazole. Assuming a molecular weight of 24,500 (see Section III) and $E^{1\%}_{1cm}$ at 280 mμ of 18.7, the spectrophotometric results give an immediate estimate of the purity of CHT-B. Following the procedure of Schonbaum et al., a 1 per cent solution of CHTG-B was activated with trypsin as described in the Appendix, and 100 μl samples were introduced into a 1 cm silica cuvette previously filled with 3 ml of 0.1 M acetate buffer, pH 5.0, and 10 μl of a suitable stock solution of cinnamoyl-imidazole in acetonitrile.* The spectrophotometer was set at 335 mμ and the chart speed was adjusted to one inch per minute. The magnitude of the drop in optical density due to acylation of the enzyme could be related to the purity of the preparation (activatability) by the formulae:

---

*Cinnamoyl-imidazole was kindly supplied by Dr. G. R. Schonbaum.

$$M_{C.I.} = \frac{A_{335}}{9.3 \times 10^3} \times 3.11, \quad M_{CHT-B} = \frac{A_{stock}}{18.7 \times 24,500},$$

$$\% \text{ purity CHT-B} = \frac{M_{C.I.}}{M_{CHT-B}} \times 100$$

where $M_{C.I.}$ are the mmoles of cinnamoyl imidazole used up during the acylation; A_{335} is the drop in optical density observed in the spectrophotometer at 335 mμ plus the change in optical density which is observed by the addition of 100 μl of enzyme to 3 ml of buffer only; M_{CHT-B} are the mmoles of enzyme acylated, and A_{stock} is the absorbancy of the stock zymogen solution at 280 mμ.

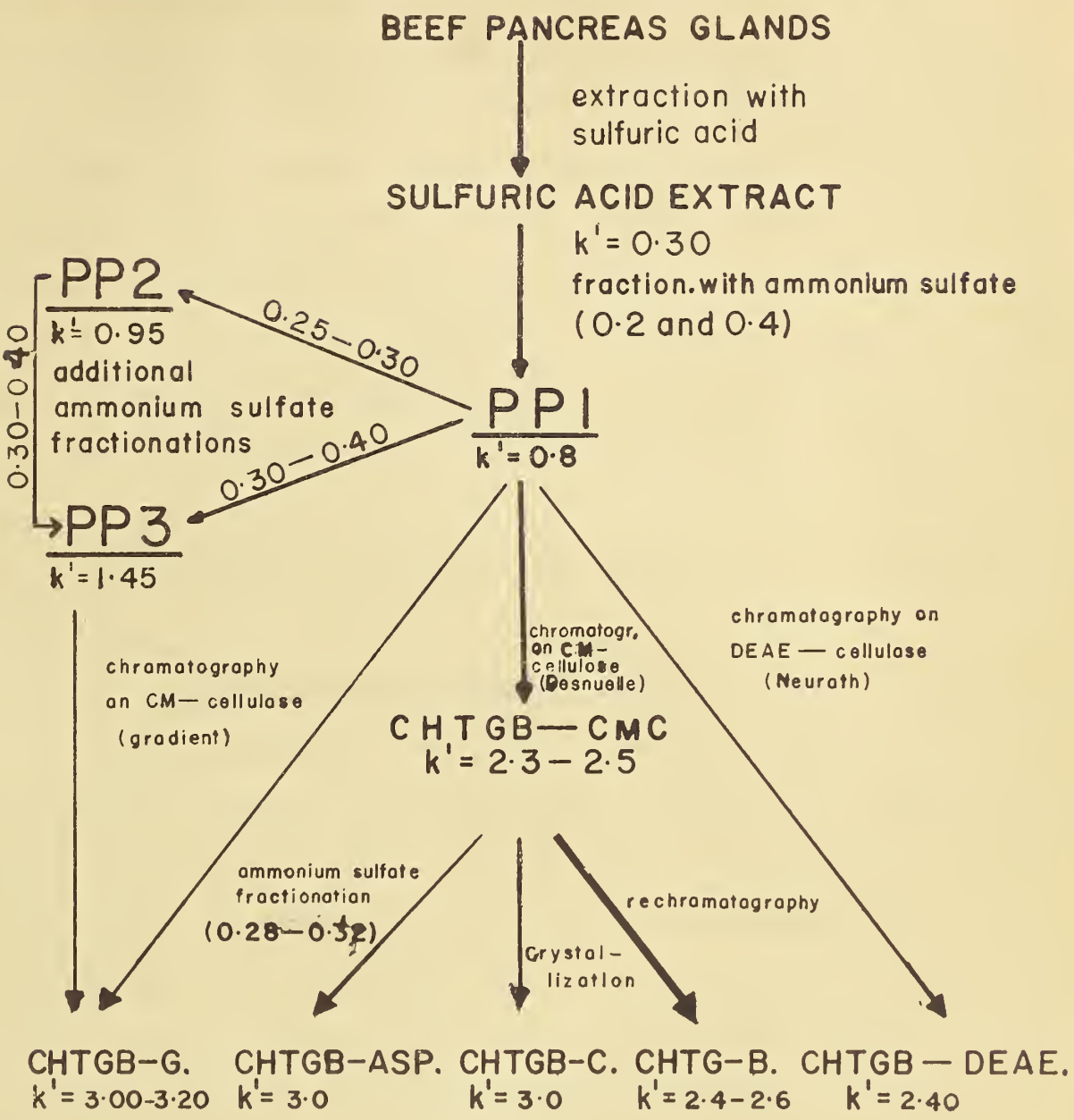
B. Results and Discussion

1. Preparation of protein for column chromatography

Table I summarizes the various approaches used for the preparation of CHTG-B. As can be seen, every batch of pancreas glands was routinely processed to the stage of PP 1 from which point the investigations of various techniques for the purification of the zymogen were started. The values of k' represent the different levels of potential chymotryptic activity determined as outlined in the Appendix. Chymotrypsinogen B - DEAE exhibited a high percentage of free activity (1.8%), while that present in all other preparations was not higher than 0.2 %. The thick arrows indicate the procedure reported by Rovery and Desnuelle (32) and will be discussed subsequently.

Table I

Methods for the Preparation of Chymotrypsinogen-B



In the procedure which has been adopted by this laboratory, an additional fractionation with ammonium sulfate was introduced to allow the separation of larger amounts of CHTG-B by one chromatographic run. Table II represents data obtained by such an experiment. It shows that this step can be justified by the achievement of a twofold purification of PP 1. While a large amount of inactive protein is removed within the limits of 0.00 to 0.25 saturation with ammonium sulfate, 41 per cent of the activity units recovered are concentrated in the last fraction. Moreover, refractionation of PP 2 together with another batch of PP 1 does not seem to give rise to deterioration, and results in an almost negligible loss of chymotryptic activity.

2. Chromatography on DEAE-cellulose

Figure 1 presents the elution diagram of CHTG-B from a preparative column. The arrow above fraction 100 indicates the start of gradient elution and the dotted line shows the linear gradient of phosphate buffer. The points on the broken line signify the potential chymotryptic activities of various fractions. Approximately 25 per cent of the total protein of PP 1 could be recovered in the pooled fractions (fractions with an absorbancy greater than 1.0), while 30 per cent was contained in the break-through peak. Although the center fraction of the peak had reasonably good potential activity ($k'=2.40$), it was found to be electrophoretically heterogeneous when tested in 0.1 M tris buffer, pH 7.5. A second peak and a shoulder moving

Table II

Additional Fractionation with Ammonium Sulfate

Limits of saturation	k'	Activity units recovered	Protein recovered
		(%)	(%)
0.00 - 0.25	0.3	9	24
0.25 - 0.30	0.95	29	19
0.30 - 0.40	1.45	41	21

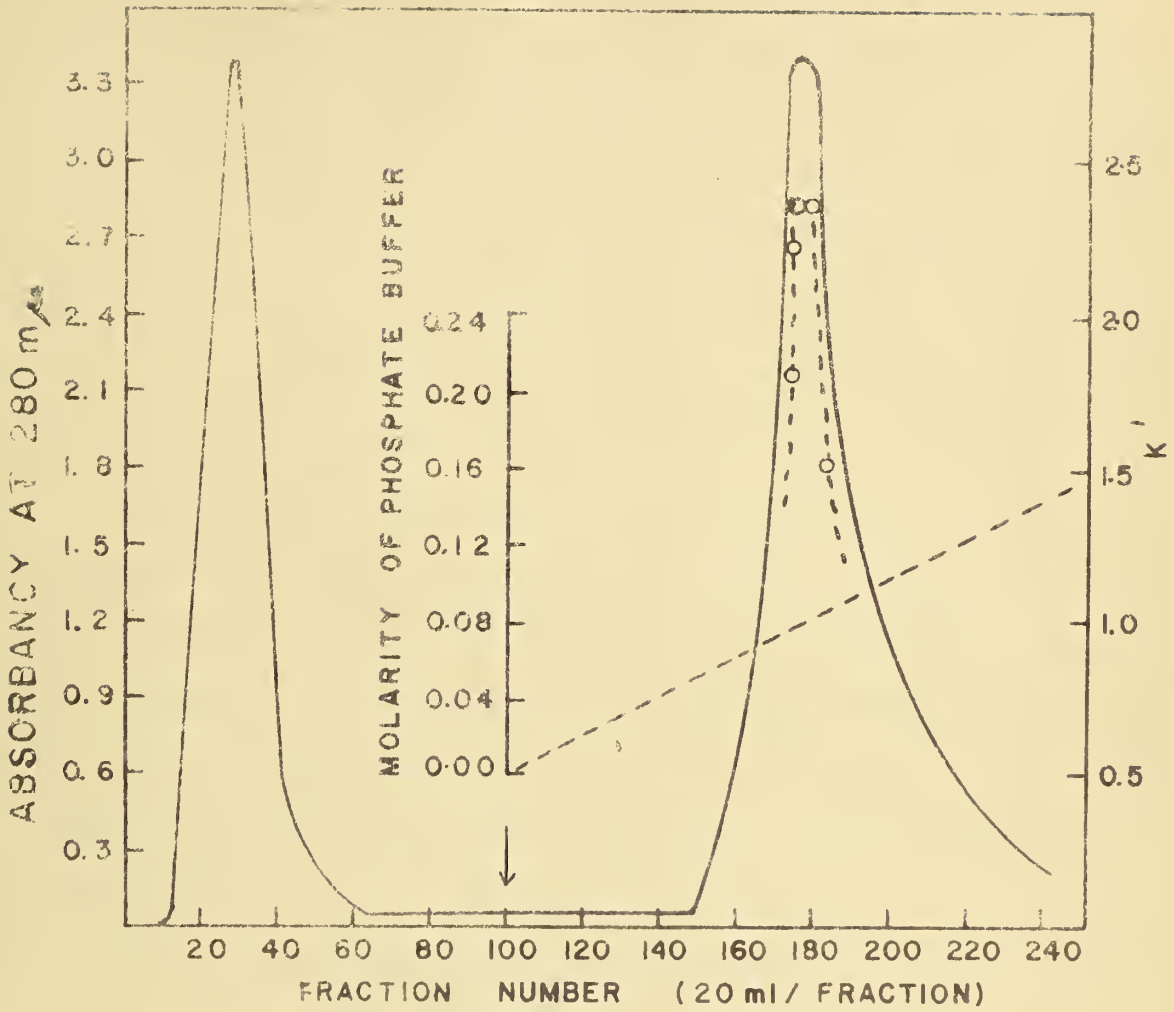


Figure 1

Chromatography of Protein Powder 1 on DEAE-Cellulose (for explanation, see text)

at a slower rate than the main component could be seen. In view of later observations (see Section 4 of this chapter) the contaminating components might have been produced by the action of free CHT-B on the zymogen at the pH of the electrophoresis experiment. This is doubtful for two reasons, however: this phenomenon did not occur at pH 7.5 with another preparation, but at pH 9.5 only, and moreover, gave rise to a faster moving peak. In the ultracentrifuge, a single peak could be demonstrated in the above buffer. The base line on the photographs appeared to be somewhat ill-defined.

Taking into consideration the above observations and the exceptionally high free activity (1.8%), it was concluded that the conditions of column chromatography on DEAE-cellulose did not render a preparation of satisfactory quality. This conclusion is also supported by Keller, Cohen and Neurath (2) who found a k'_{m}=1.53 for anionic component 2 after separation on DEAE-cellulose under identical conditions.

3. Chromatography on CM-cellulose

a. Stepwise elution according to Rovery et al. (32)

The results to be described in the following paragraphs were obtained during a study which arose from the observation made in this laboratory that the method reported by the above authors did not yield the expected results. Early experiments indicated that CHTG-B could not be eluted from CM-cellulose with 0.05 M sodium citrate buffer, pH 4.6. It is now known that this is only partially true. Table III will serve to

Table III

Chromatography of Protein Powder 1 on CM-Cellulose

Capacity of CM-cellulose resin	Molarity of the eluting buffer					
	0.05 M citrate		0.06 M citrate		0.07 M citrate	
	Recovery	k'	Recovery	k'	Recovery	k'
(meq)	(%)		(%)		(%)	
0.5	40	1.8	Not done		48	1.3
0.59	9.0	-	30-40	2.55	45	2.0
0.67	Protein not eluted		Protein not eluted		1	-
0.9	- - - Small yield with 0.2 M sodium citrate buffer - - - - -					

illustrate the point. It summarizes the results obtained from a number of chromatographic runs performed on CM-cellulose columns of varying capacity. Using resin with a capacity of 0.9 meq per g, the zymogen could be eluted by sodium citrate of high ionic strength (approximately 0.2 M) only. The yield under these conditions was found to be very low. On resins with lower capacities the protein was bound less strongly and could be eluted in good yields by sodium citrate buffers having molarities close or identical to that reported by the French workers. It becomes also evident that resin with a capacity of 0.59 meq per g and 0.06 M citrate buffer, pH 4.6, yielded the best preparation. It was thereby possible to reproduce the findings previously reported.

Figure 2 presents a typical elution diagram of an analytical CM-cellulose column, capacity 0.59 meq per g. The arrow indicates the point of change to 0.06 M sodium citrate buffer, pH 4.6. The large peak represents CHTG-B - CMC and the broken line inside the peak shows the potential chymotryptic activity of various fractions. Since CHTG-A, trypsinogen, and ribonuclease are strongly adsorbed at this pH, only a very small break-through peak emerges from the column (approximately 5%). The value of 2.55 for the k'_{max} of the center fractions agrees well with that reported by Rovery ($k'=2.5$). It is of interest to note that in both cases the front of the peak is composed of the more active fractions whereas the trailing edge contains material of lesser quality. In contrast to Rovery who claims

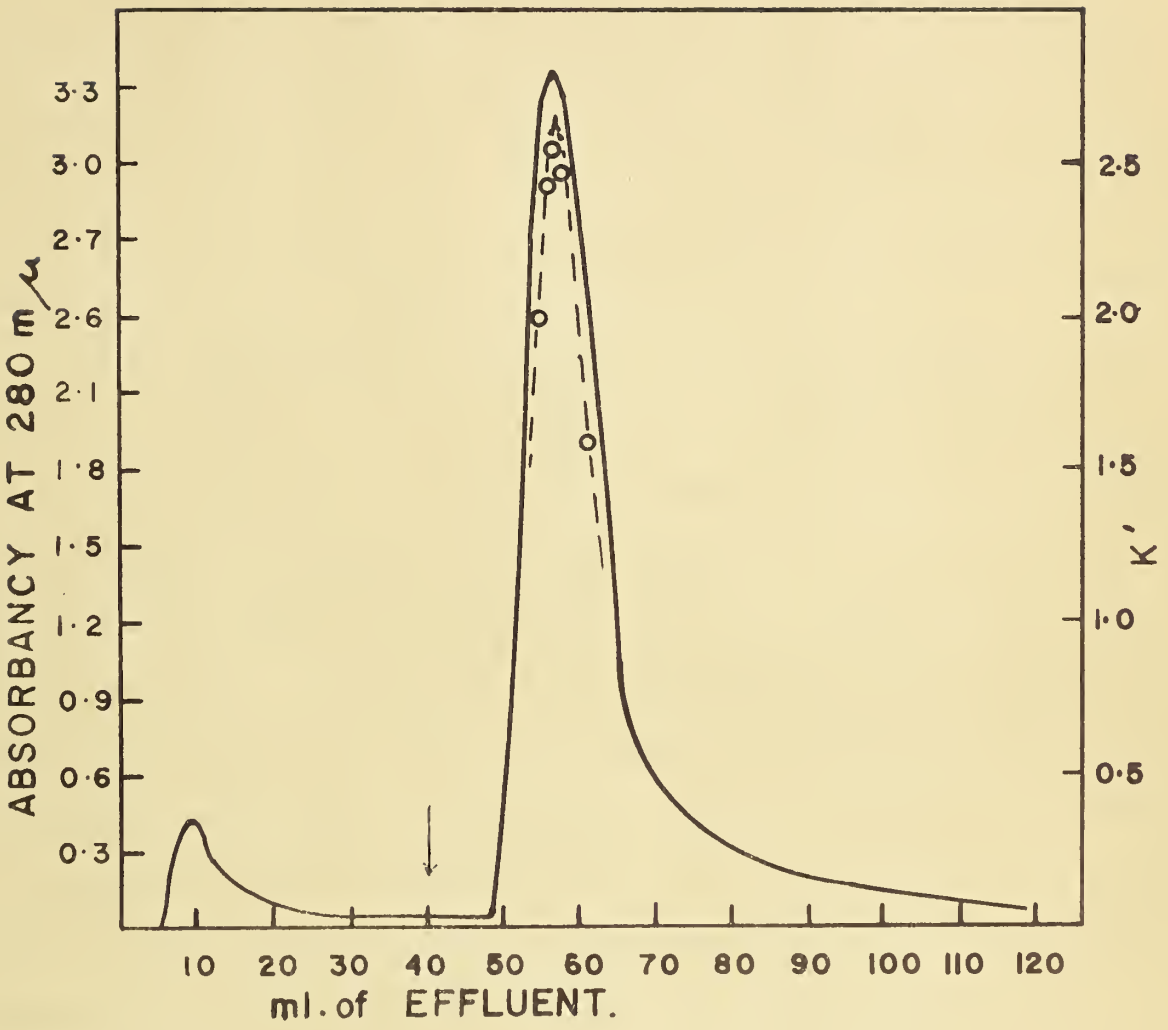


Figure 2

Chromatography of Protein Powder 1 on CM-Cellulose, stepwise elution (for explanation, see text)

Recovery of protein under main peak, 20%.
Recovery of activity, 50%.

to have prepared zymogen devoid of free activity, zymogen in this laboratory invariably contained up to 0.1 per cent of free activity. It is believed that a decreased inhibitory efficiency of DFP at pH 4.6 is responsible for these findings. This view is supported by the observation that the free chymotryptic activity of a 2 per cent solution of CHTG-B is inhibited to the extent of only 30 per cent by a 30-minute incubation with 0.0001 M DFP in 0.1 M acetate buffer, pH 5.5.

Chymotrypsinogen-B - CMC could be further purified by two methods. Crystallization as described in Methods produced a 17 per cent increase in potential chymotryptic activity (CHTG-B - C, $k' = 3.0$). Unexpectedly, the potential activity of the dialyzed and lyophilized mother liquors was identical to that of the crystals. Furthermore, a considerable loss of protein from the dialysis bag took place during the crystallization procedure. Presumably, this arose from the breakdown of inactive or denatured protein since the activity units could be recovered almost quantitatively. The fractionation of CHTG-B - CMC with ammonium sulfate between 0.28 and 0.32 saturation led to an equally active preparation (CHTG-B - ASF, $k'=3.0$). Since both of these preparations were used extensively for physico-chemical as well as chemical studies, a discussion of their homogeneity is presented in Section 4 of this chapter.

b. Gradient elution with sodium citrate buffer, pH 4.47

In the studies on the stepwise elution of CHTG-B by the

method of Rovery and Desnuelle, it was observed on several occasions that the pH of the effluent at the time of elution of the main protein peak was 4.5. Since gradient elution would be expected to give a protein preparation of higher homogeneity, a method was developed using a linear gradient of sodium citrate buffers at a pH close to 4.5. It was found that this procedure would, in fact, give products of high potential activity ($k'=3.0$ to 3.2) and low free activity (0.01 to 0.03%. Based on optical density measurements, recoveries of protein under the main peak from PP 1 and PP 3 were 25 per cent and 35 per cent respectively. The chromatographic procedure on a 6 cm column could be completed in six hours. The top half of Figure 3 presents the elution diagram of a 3 cm x 50 cm column charged with 400 mg of PP 1 in 20 ml of 0.03 M sodium citrate buffer, pH 4.47, containing 0.005 M DFP. Several fractions between tube number 48 and 114 were tested for potential chymotryptic and nucleolytic activity. From the bottom half of Figure 3, it becomes evident that protein exhibiting potential chymotryptic activity emerges from the column in three distinct peaks. Chymotrypsinogen-B, the main component of peak one showed maximal activity in the center fractions ($k'=3.2$) when tested with ATEE after tryptic activation. Since the concentration in each tube was different, the conditions of activation were clearly not identical for each effluent fraction. Nevertheless, all gradient runs analyzed in the same way showed a very sudden increase to maximal values

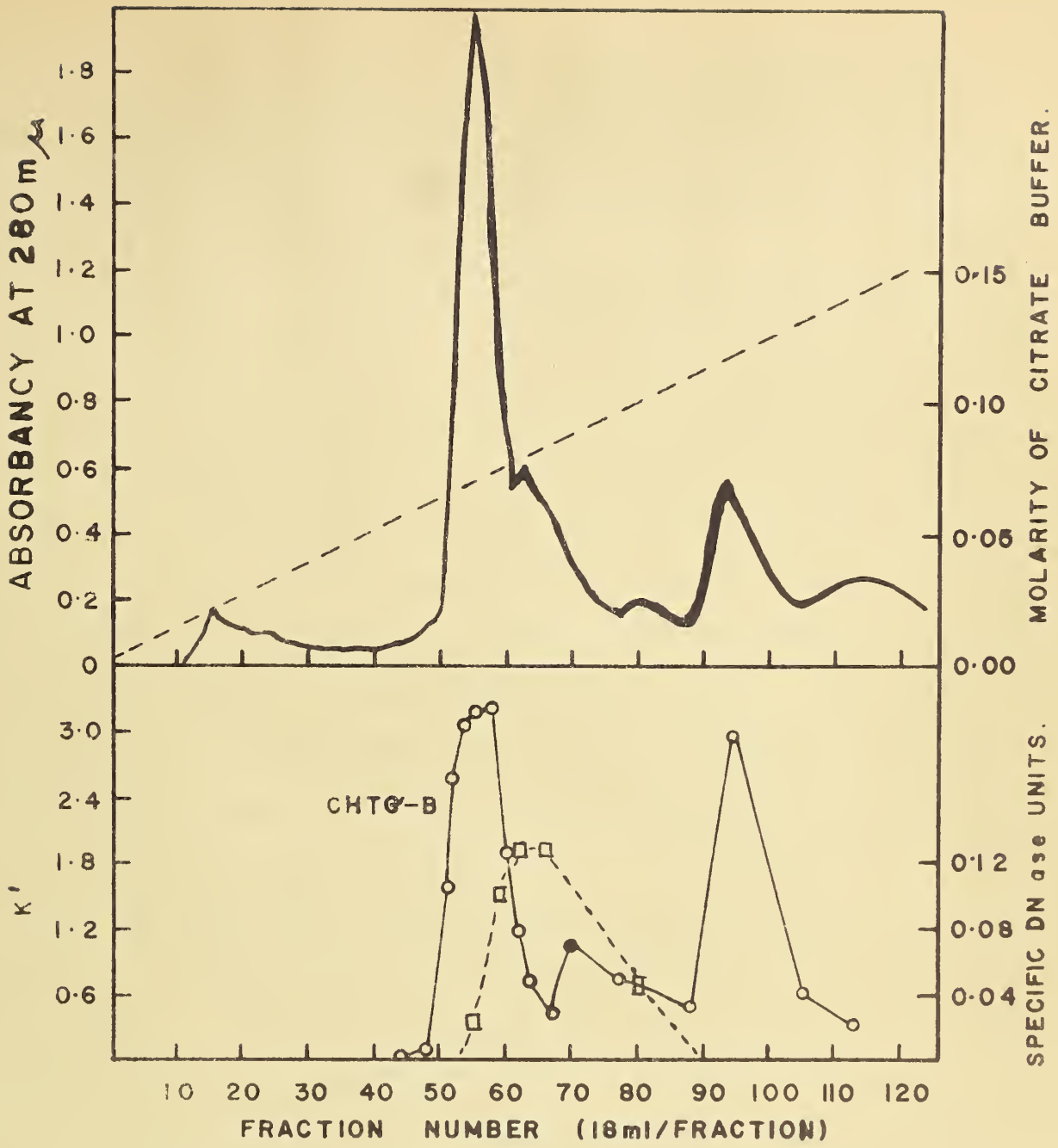


Figure 3

Chromatography of Protein Powder 1 on CM-Cellulose, Gradient Elution
Top half - elution diagram.
Bottom half, open circles - activity after activation of fractions versus ATEE; open squares - activity of fractions versus deoxyribonucleic acid - methyl green.
(For explanation, see text)
Recovery of protein in tubes 50-62, 22%.
Recovery of activity, 65%.

of k' at the front edge of the peak. The trailing side contained zymogen with lower activity for reasons which will become apparent from the following paragraphs and were not included in the pooled fractions.

It was shown by Laskowski and Kassel (28) that CHT-B could be separated from the zymogen on CM-cellulose columns by changing the pH of the eluting buffer from 4.5 to 6.0. In order to determine the elution characteristics of CHT-B on the gradient system, the following experiment was conducted. Twenty-one mg of protein consisting of PP 1 (50%) and activated PP 1 (50%) were charged on a 0.9 cm x 43 cm column and subjected to gradient elution. Figure 4 shows the results. The open circles represent potential chymotryptic activity and the full circles denote free activity. Although CHT-B does not form a sharp boundary in this particular case, it is eluted after the zymogen and does not contaminate the inactive form to any great extent. It should also be mentioned that there was ample time for autolysis. Prior to application to the column the activated solution of PP 1 (pH 8.0) was dialyzed for 24 hours against 0.03 M citrate buffer to lower the pH to 4.47. This treatment could well account for the low specific activity of CHT-B. From the results of the above experiment it can be concluded that the second peak of chymotryptic activity is in all probability contributed by CHT-B, although there still exists the possibility that neochymotrypsinogen(s) is concentrated in these fractions.

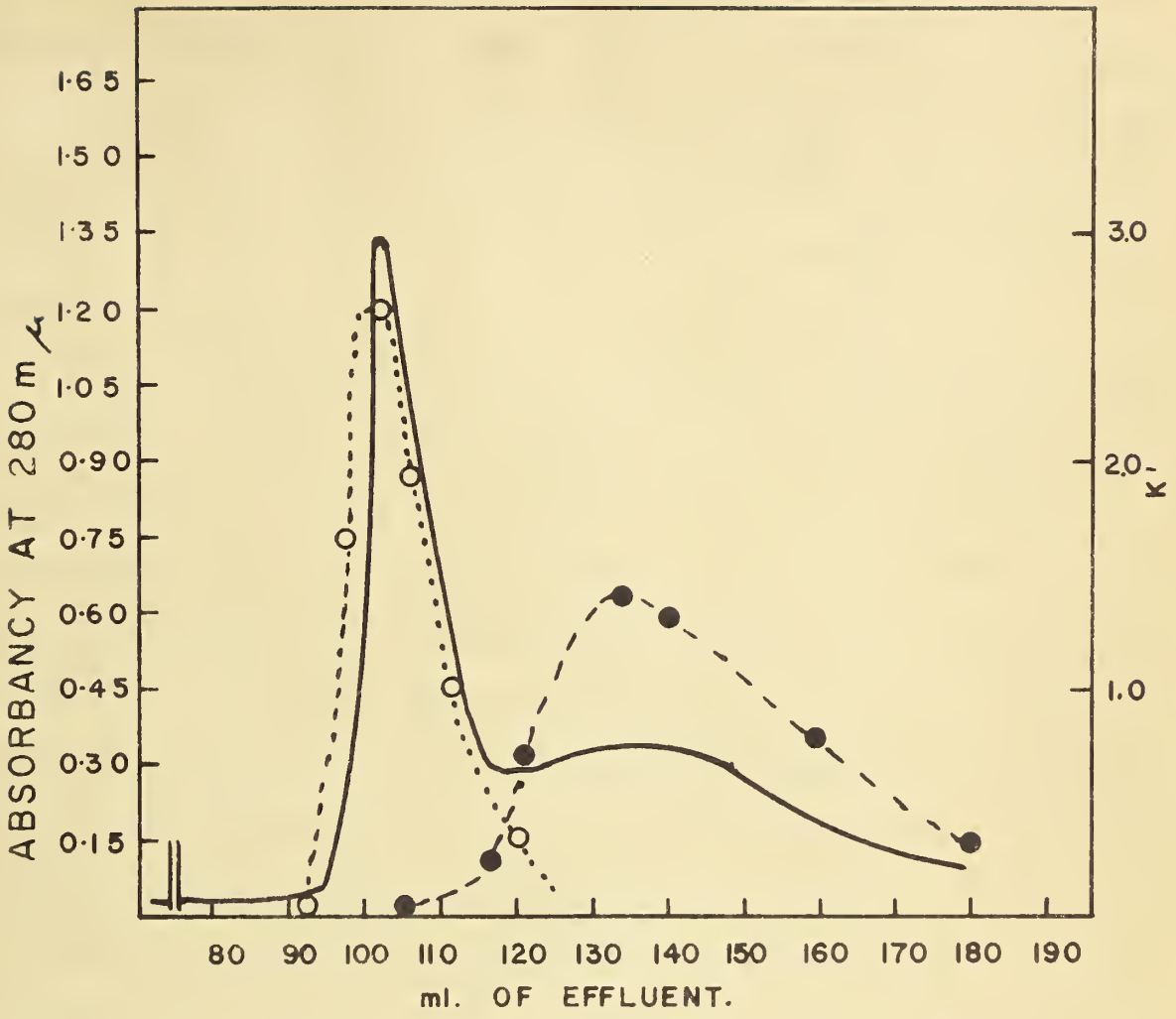


Figure 4

Separation of CHTG-B and CHT-B on CM-Cellulose, Gradient Elution (for explanation, see text)

When fraction 94 was tested for its ability to hydrolyze ATEE, substantial activity was observed. It is possible that CHTG-A is concentrated in the last peak since Maroux, Rovery and Desnuelle (49) have shown that it could be eluted from CM-cellulose with 0.08 M ammonium acetate buffer, pH 6.0. The protein contained in fractions 90 to 100 has not been further characterized.

Kunitz presented evidence in 1950 that deoxyribonuclease I (DNase I) present in the sulfuric acid extract of beef pancreas glands, could be precipitated with ammonium sulfate between 0.2 and 0.4 saturation (4). As early as 1946, Laskowski observed that his preparations of CHTG-B were contaminated by protein with nucleolytic activity (21). It was therefore possible that DNase was present in our preparations of CHTG-B. When tested by the method described in the Appendix, approximately 3 per cent DNase was shown to be present in PP 1. It was then of interest to investigate the possibility of any of the effluent fractions containing DNase. The open squares drawn on the bottom half of Figure 3 indicate that protein with activity against deoxyribonucleic acid is eluted from the column between CHTG-B and CHT-B. Assuming the specific activity of pure DNase to be 1.0 (50), the contamination of fractions 62 and 66 amounts to approximately 13 per cent, and to 2 per cent in fraction 55 which contains the proteolytic zymogen. When a number of freeze-dried preparations of CHTG-B were tested, 1-2 per cent of nucleolytic activity could be detected.

4. Moisture content, per cent nitrogen, and $E^{1\%}_{1\,cm}$ at 280 mμ

On a dry weight basis (CHTG-B - G used was found to contain 9 per cent moisture), total N as determined by the Kjeldahl method was 16.06 ±0.06%. The extinction coefficient of a 1 per cent solution of CHTG-B - G in 0.001 M HCl was calculated as 18.7.

5. Homogeneity of CHTG-B - C, G, and ASF

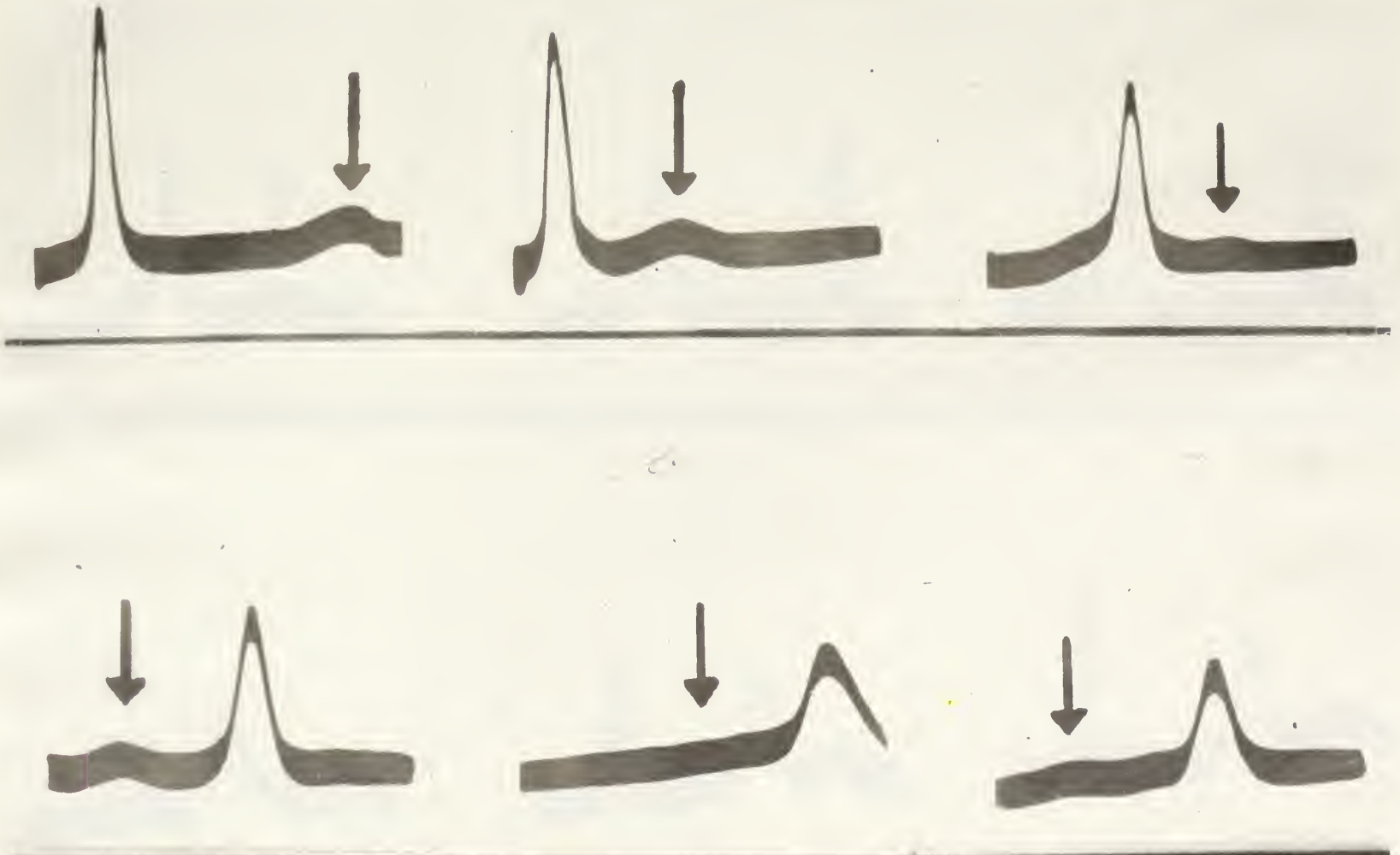
In order to demonstrate the absence of contaminating protein other than deoxyribonuclease, preparations of CHTG-B were subjected to various tests. These included ultracentrifugation, electrophoresis, C-terminal analysis, assays with cinnamoylimidazole, and studies with various substrates.

a. Ultracentrifugation

In all sedimentation-velocity experiments reported in Section II.B.2, a single symmetrical peak could be observed. The molecular weights determined at the meniscus and cell bottom of Archibald runs were similar and did not reveal any tendency to change as a function of time. All preparations tested yielded almost identical values for the molecular weight.

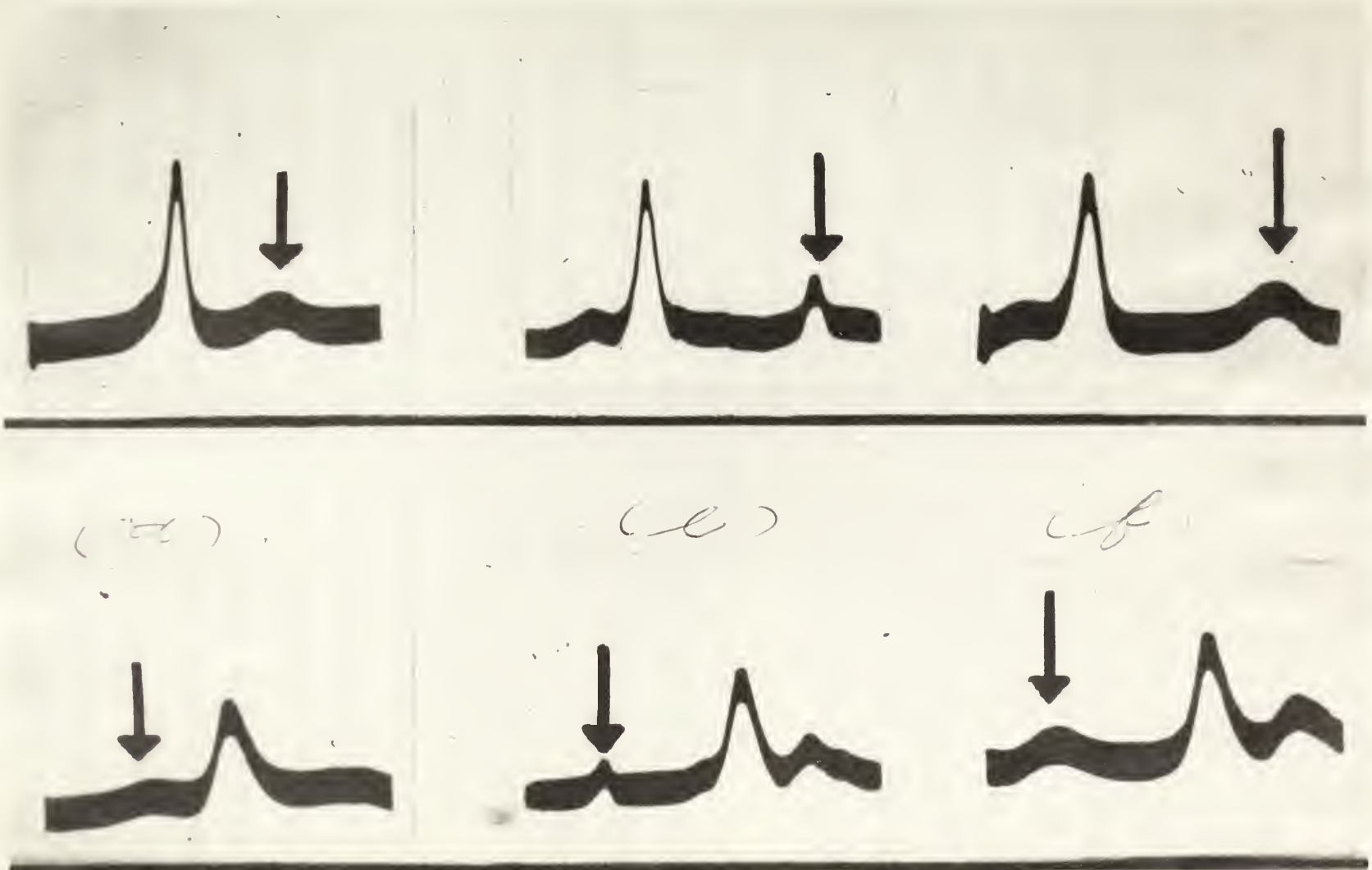
b. Electrophoresis

CHTG-B - C was employed exclusively. Plates A and B show representative photographs of six different runs performed in various buffers, ionic strength 0.1, which covered the pH range from 3 to 9.5. Exact details of each run are collected in the Appendix. In all but two cases, a single peak was observed.



Electrophoretic patterns of CHTG-B. Top row represents photographs of ascending limb, bottom row of descending limb. (a) pH 2.96, (b) pH 4.2, (c) pH 5.8.

Plate A



Electrophoretic patterns of CHTG-B. Top row represents photographs of ascending limb, bottom row of descending limb. (d) pH 7.85, (e) pH 9.85, (f) pH 4.5.

Plate B

At pH 9.48, a second component (Plate B, e) migrating at a somewhat faster rate than the main peak was visible. This component could arise from an impurity or be formed by the action of free CHT-B on the zymogen or through denaturation at high pH. To check the latter possibilities, CHTG-B - C was incubated at pH 9.48 and 0°C for 12 hours followed by dialysis against 0.1 M acetate buffer, pH 4.5. On electrophoresis, the two peaks shown in Plate B, f, were observed. Although it could not be decided if the second component was produced by denaturation or autolysis, it was demonstrated that the treatment with pH 9.48 buffer was responsible for the observed heterogeneity.

c. C-terminal analysis

It was shown by Kassel and Laskowski that the C-terminal amino acid of S-sulfochymotrypsinogen-B was asparagine (38). From native CHTG-B no terminal amino acid was liberated by carboxypeptidase-A in molar ratios. Nevertheless, detectable amounts of various amino acids were produced from Laskowski's and Rovery's preparations when treated with a high ratio of the exopeptidase (28,34). Laskowski suggested that the findings of C-terminal tyrosine were indicative of chymotryptic autolysis. It was with this statement in mind that the investigation of the C-terminal amino acids was undertaken.

Table IV shows the molar quantities of amino acids liberated from several preparations of CHTG-B during a one-hour digestion period. It can be seen that CHTG-B - C has undergone

Table IV

Liberation of Amino Acids by Carboxypeptidase A

Preparation	μmoles of amino acids liberated per μmole of CHTG-B					
	leucine	valine	phenyl-alanine	alanine	tyrosine	threonine
CHTG-B-ASF	0.29	0.12	0.09	0.08	0.07	0.04
CHTG-B-C	0.40	0.12	0.27	0.09	0.31	0.36
CHTG-B-G	0.09	0.07	0.03	0.06	trace	trace
	0.09	0.08	0.06	0.05	trace	trace
	0.07	0.06	-	0.03	trace	trace

considerable proteolysis. Whereas the findings of high levels of tyrosine and phenylalanine did not come as a surprise, the large amounts of leucine liberated were appreciated only after the ability of CHT-B to hydrolyze the leucyl peptide bonds of glucagon had been demonstrated (see Section IV.B). The results from CHTG-B - G indicate only very limited proteolysis. Results from several time studies suggest that valine, alanine, and perhaps even phenylalanine are released after leucine and may well constitute a sequence with leucine being the C-terminal amino acid. A sequence of very similar nature is known for CHTG-A (12). CHTG-B - ASF appears to occupy an intermediate position between the other two preparations and comes closest to the results reported by Laskowski (28).

In conclusion, the data indicate that the autolysis of chymotrypsinogen-B during gradient elution chromatography on CM-cellulose is not extensive.

d. Assay with cinnamoyl-imidazole

Figure 5 presents the spectrophotometric record of the reaction between 100 µl of a 1.07% solution of CHT -B and cinnamoyl-imidazole in 0.1 M acetate buffer, pH 5.0, and 25°C. At A, 10 µl of cinnamoyl-imidazole (molar extinction coefficient 9.3×10^3) was added, resulting in an increase of optical density by 0.490 units (A'). Curve A'B represents the spontaneous hydrolysis of cinnamoyl-imidazole at pH 5.0. At B, 100 µl of the enzyme solution was added and the absorbancy

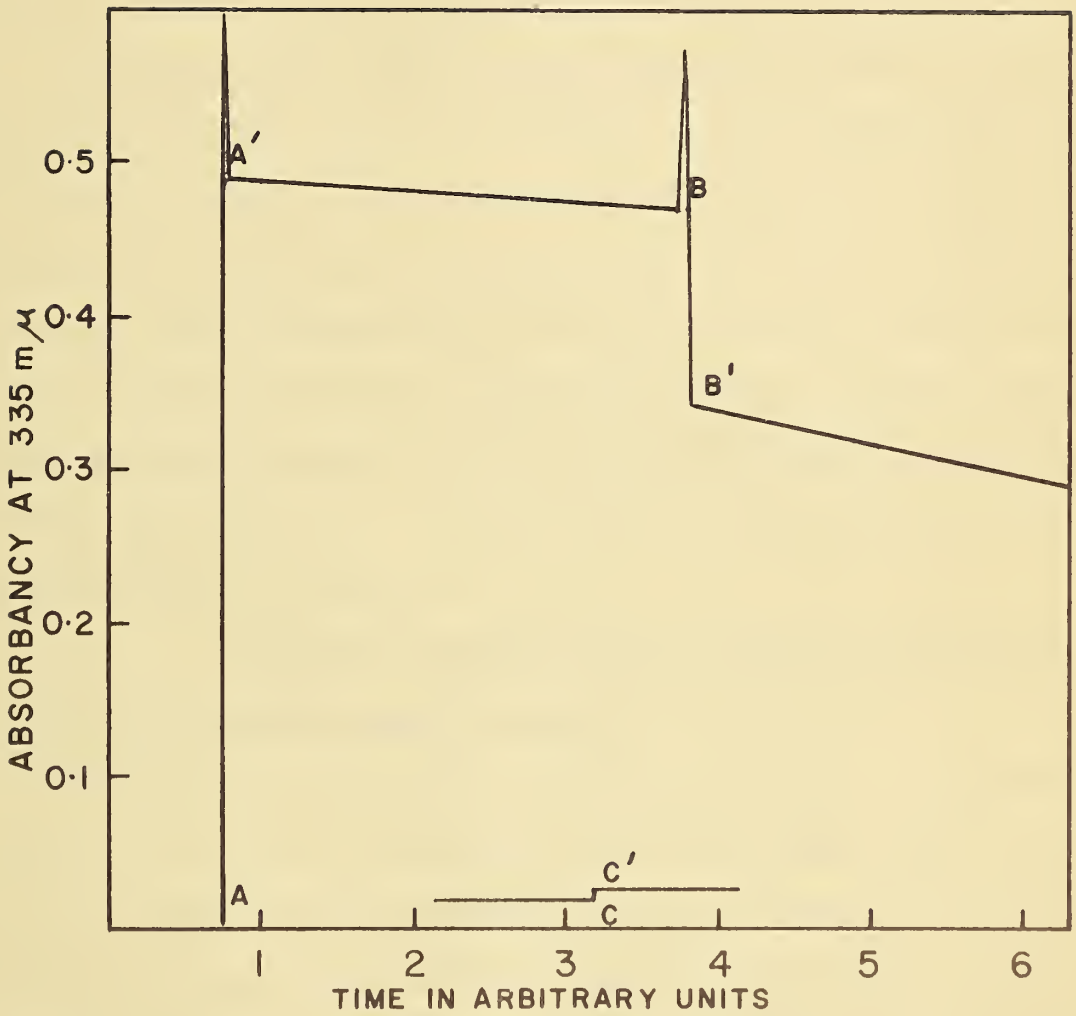


Figure 5

Reaction of Cinnamoyl-Imidazole with CHT-B; Spectrophotometric Record (for explanation, see text)

dropped to B' due to acylation. From B', enzymic as well as spontaneous hydrolysis of cinnamoyl-imidazole determine the slope of the curve. At C, 100 µl of the same enzyme solution was added to buffer alone. When these data were introduced into the equations given on page 25, the enzyme was calculated to be 93.1 per cent homogeneous. Tests in the pH-stat revealed a value of 3.13 for the k'.

Preparations of CHTG-B were routinely tested by this assay and showed 85 to 95 per cent purity. All calculations done point to a value of 3.36 as the k' for a 100 per cent pure preparation which makes fractions 54 to 58 approximately 90 to 95 per cent homogeneous. It is, of course, obvious that the above determinations are only meaningful if accurate measurements of the molecular weight and the extinction coefficient of CHTG-B are available. Special care was taken in the determination of these parameters.

e. Evidence against the presence of a separate enzyme hydrolyzing leucine peptide bonds

As will be seen in Part IV of this thesis, comparative studies of digests of glucagon with CHT-A_4 and CHT-B revealed that the latter enzyme hydrolyzed peptide bonds involving the carboxyl group of leucine. No evidence for such activity was found in the case of CHT-A_4. It was of interest to investigate the leucine peptidase activity in order to determine whether such activity was an inherent property of CHT-B or whether it was due to contaminating protein. As a synthetic substrate,

benzoyl-L-leucine-ethyl-ester (BLEE; for preparation, see Chapter IV.A) was chosen. In the following paragraphs, several lines of evidence are discussed which indicate that the leucine peptidase (or esterase) activity is an inherent part of the chymotryptic B activity.

(i) Leucine esterase activity requires activation with trypsin and the zymogen does not exhibit any activity against BLEE. Furthermore, both activities reach the maximal value after less than 10 minutes as determined by the following experiment. Two samples of CHTG-B - G were dissolved in 0.1 M Tris buffer, pH 8.0. Aliquots were withdrawn and checked for activity against ATEE and BLEE. The zymogen showed 0.02 per cent of free activity as determined by the hydrolysis of ATEE and none could be detected against BLEE. Both samples were activated and aliquots were withdrawn after 10 and 30 minutes of activation. When tested against both substrates, the following values of k' were evaluated: k' for the hydrolysis of ATEE was 2.98 and 2.95 after 10 and 30 minutes, respectively; k' for BLEE was 0.034 and 0.034.

(ii) Diisopropylfluorophosphate inhibits leucine esterase activity to the same extent as the activity versus ATEE. This was shown by incubation of a 1 per cent solution of CHT-B in 0.1 M Tris buffer, pH 8.0, containing 0.0001 M DFP. Aliquots were withdrawn after 5, 10, 20, and 60 minutes of reaction and tested against ATEE and BLEE. Tests with both substrates indicated that CHT-B retained 30, 12, 1.5, and 0.5 per cent of activity, respectively.

(iii) The ratio of k' for the hydrolysis of BLEE to the k' for that of ATEE remains constant and equal to $1.15 \pm 0.05 \times 10^{-2}$ from step to step of the preparative procedure. Checks were made on the following protein preparations with the ratios given in parentheses: PP 1 (1.17×10^{-2}); PP 3 (1.14×10^{-2}); CHTG-B - C (1.13×10^{-2}); CHTG-B - G (1.20, 1.12, 1.10×10^{-2}); and CHTG-B - ASF (1.14×10^{-2}).

(iv) The ratio of k' for ATryEE/k' for BLEE remains constant within the limits of error when evaluated on seven different fractions of a chromatographic peak of CHTG-B - G. Table V shows the results of such an experiment. Together with the number of each fraction, the absorbancy at 280 mμ is given. Although the agreement between the ratios cannot be considered perfect, no trend can be detected. It also has to be mentioned that it becomes increasingly difficult to evaluate the ratios accurately as the concentration in the fractions decreases. Large quantities of enzyme solution have to be used in order to produce a measureable reaction rate.

(v) Indole, a competitive inhibitor for CHT-A_4, inhibits both the leucine esterase activity of CHT-B and the activity versus ATEE, to the extent of 80 and 50 per cent, respectively. This would be expected since BLEE represents a poorer substrate for the B enzyme than ATEE.

(vi) When CHT-B which had been isolated by gradient elution of CM-cellulose columns was used for the digestion of glucagon, the peptide bonds of leucine were again hydrolyzed.

Table V

Activity Ratios of Column Fractions Against ATryEE and BLEE

Fraction Number	Absorbancy (280 mμ)	k'_{ATryEE}/k'_{BLEE}
1	0.128	17
2	0.685	20
3	1.030	19
4	1.000	20
5	0.642	20
6	0.345	21
7	0.319	23

Assuming for the moment that the leucine esterase activity was not an intrinsic component of the chymotrypsin-B enzyme but was due to a contamination, the results of the above experiment could be interpreted only by assuming that the inactive and activated leucine esterase behaved identically to CHTG-B and CHT-B, respectively, during gradient elution from CM-cellulose.

C. Conclusions

Highly homogeneous CHTG-B can be prepared by gradient elution of CM-cellulose columns at pH 4.47. The preparation is 90 to 95 per cent pure as judged by the cinnamoyl-imidazole assay and shows a single peak in the ultracentrifuge and the electrophoresis apparatus. Only small quantities of C-terminal amino acids are liberated by carboxypeptidase-A (molar ratio of zymogen to carboxypeptidase, 12 to 1), a criterion which demonstrates the superiority of gradient elution over the other techniques investigated in this laboratory. When CHTG-B, prepared in this laboratory, is compared with properties of the zymogen as prepared in other laboratories, the superiority of the gradient procedure is clear. Zymogen, prepared by Rovery et al. (32), exhibits a zero order rate constant of hydrolysis of ATEE (k') of 2.70 (corrected from 2.60 for $E^{1\%}_{1\,cm} = 18.7$ at 280 mμ) representing 80 per cent homogeneous protein. No complete results on the C-terminal amino acids are available at the present time. In Laskowski's laboratory, chymotryptic activity is measured with casein as substrate. Results from

this assay cannot be compared to pH stat measurements directly. Because of possible variations in the nature and quality of the casein preparation used in this assay, experiments which were designed to arrive at a conversion factor gave inconclusive results. However, analysis of C-terminal amino acids (28) tend to indicate that crystallization as used for the preparation of CHTG-B promotes autolysis.

III. PHYSICO-CHEMICAL STUDIES ON CHYMOTRYPSINOGEN-B

At the beginning of this work, only one estimate of the molecular weight of CHTG-B was available (21,600 by sedimentation-diffusion (36)). In 1961, Kassel and Laskowski published the results of a complete amino acid analysis of the zymogen and evaluated a minimum molecular weight of 23,086 (29). The lack of data prompted the reinvestigation of this problem. It was aimed to employ a number of physico-chemical techniques and to arrive at an accurate value for the molecular weight of CHTG-B which would serve as a basis for studies on the purity of the zymogen preparations (see Section I.B) as well as for further chemical characterizations. For these reasons, CHTG-B has been characterized from measurements of its sedimentation rate, intrinsic viscosity, light scattering, and approach to sedimentation equilibrium.

A. Methods

Throughout these studies various samples of CHTG-B were used. Since the physico-chemical parameters of the zymogen were not dependent on the method of preparation as long as the activity was high ($k'=2.95$ or higher), no reference will be made to any particular preparation in the description of methods.

1. Determination of the isoelectric point

Electrophoresis experiments were performed in a Perkin-Elmer apparatus (Model 38a) equipped with a modified Philpot-Svenson cylindrical lens system. The runs were carried out in a 2 ml Tiselius cell at 0°C. and photographs were taken on Kodak sheet film, using the film adaptor contained in the viewing hood. The details for each of the electrophoresis experiments have been given in the Appendix. To determine the electrophoretic mobility (μ) of CHTG-B in different buffer solutions, the number of amperes of current (I) were recorded for each run and the specific conductance (k) of each protein solution was measured in a standard conductivity cell. The distance in cm (d) moved by the boundary in t seconds was measured* and introduced into the following equation:

$$\mu = \frac{d \cdot k \cdot A}{I \cdot m \cdot t} ,$$

where A is the cross sectional area of the Tiselius cell and m the magnification factor of the camera. The values for the mobility were plotted against pH and joined by straight lines. The pH of zero mobility was taken as the isoelectric point.

Solutions (0.8%) of CHTG-B in buffers of 0.1 ionic strength and varying pH were prepared and used immediately. Table VI presents a list of buffers together with the corresponding pH

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*The pattern of the descending limb was used for measurements only.

Table VI

Buffer Systems for Free Boundary Electrophoresis

pH	Buffer system
3.0	glycine (0.1M) - HCl (0.1 M)
4.5	acetic acid - sodium acetate (total ionic strength = 0.1)
6.0	cacodylic acid - sodium cacodylate (total ionic strength = 0.1)
7.5	tris (hydroxymethylamino) methane - HCl (0.1 M)
9.5	glycine (0.1 M) - NaOH (0.1 M)

values at which they were used. At the end of each run, the protein solution was recovered and the pH as well as its conductivity were determined.

2. Evaluation of the partial specific volume ($\bar{v}$)

The determination of the molecular weight of a protein requires the accurate evaluation of $(1-\bar{v}\rho)$. The partial specific volume does not vary significantly from protein to protein and lies within the range of 0.70 to 0.75 ml/g. Since the solution density (ρ) is close to unity for all commonly employed buffer systems, an error of 1 per cent in the determination of $\bar{v}$ results in a 3 to 4 per cent error in the quantity $(1 - \bar{v}\rho)$ and therefore in the final calculation of molecular weights. The partial specific volume was determined by density measurements using a pycnometer equipped with two ground glass joints, into one of which fitted a mercury thermometer calibrated to 0.05°C. Onto the other, a cap containing a small capillary hole could be slipped. Solutions of CHTG-B in 0.1 M NaCl - 0.001 M HCl were clarified by filtration through millipore filters (pore diameter 0.47 μ (51)) and the concentration (c) of each was determined by its absorbancy at 280 mμ. Demineralized water and solvent were clarified as well before being used. A water bath kept at 20 ±0.05°C. was used for the equilibration of the pycnometer and its contents. The density of the solvent and protein solutions of varying concentrations were calculated from weight measurements

and the volume of the pycnometer, and introduced into the following equation:

$$V_{app} = \frac{1}{d_o} - \frac{1}{c}\left(\frac{d - d_o}{d_o}\right) \qquad (52),$$

where V_{app} is the apparent specific volume; d and d_o are the densities of the protein solution and the solvent, respectively; and c is the concentration in grams per ml. The average value of V_{app} over the concentration range 14.4 to 6.4 mg per ml was evaluated and assumed to represent $\bar{v}$, since it is independent of protein concentration.

3. Intrinsic viscosity of CHTG-B

Ostwald-Fenske viscometers requiring 5.0 ml and with a water flow time of 480 seconds at 20°C. were used to measure the relative viscosity of CHTG-B solutions of varying concentrations in 0.1 M NaCl - 0.001 M HCl, pH 3.0. All measurements were carried out in a constant temperature bath kept at 20±0.05°C. The intrinsic viscosity $[\eta]$, was determined by plotting the reduced viscosity ($(\eta_{rel}-1)/c$) versus c (concentration in g/ml) and extrapolating the best straight line to zero protein concentration. The method of least mean squares was used to calculate the intercept and standard deviations.

4. Determination of the sedimentation constant

The variation of $s_{20,w}$ with protein concentration and pH was investigated. The buffers used were the same as outlined in Table VI. In addition, the system 0.1 M NaCl - 0.001 M HCl,

pH 3.0, was also employed. A typical evaluation of $s_{20,w}$ as a function of protein concentration will be described in the following. All the other experiments were conducted in the same manner. Forty-four mg of CHTG-B were dissolved in 2 ml of the appropriate buffer. One hundred µl of the stock solution was taken for the determination of the optical density after the pH had been readjusted to the original value; this was necessary because any solution containing CHTG-B had been dialyzed routinely against 0.001 M HCl prior to lyophylization, and solution of the freeze-dried protein resulted in a drop of the pH. Suitable dilutions were made and stored in the refrigerator until used. Each solution was analyzed in the analytical ultracentrifuge (Spinco Model E) at 59,780 rpm. All sedimentation patterns were photographed on Kodak metallographic plates with a 6-second exposure time using the schlieren optical system. Photographs were taken at zero time and after suitable time intervals (8 or 16 minutes). After development, the plates were measured on a microcomparator and the sedimentation constants were calculated from the slope of a plot of the log of the distance of the boundary from the axis of rotation against time. An allowance of 0.02 cm was made for the stretching of the rotor at 59,780 rpm (53). The temperature of the rotor was recorded throughout each run by the RTIC unit and the sedimentation constants were corrected to the density of water at 20°C., as described by Svedberg and Pederson (54).

5. Molecular weight of CHTG-B by the approach to sedimentation-equilibrium

All runs were observed in the Spinco Model E analytical ultracentrifuge equipped with a slow-speed attachment (1:3 speed reduction). A speed of 12,590 rpm was found satisfactory in most cases. However, on one occasion the ultracentrifuge was operated at 8,766 rpm. The schlieren optical system was employed with a phase plate as the diaphragm. Pictures of all runs were taken at bar angles of 60° and 75°; for measurements, the 75° angle position was found to yield the sharpest outlines. Runs were made with 12 mm cells using a 4° sector Kel-F centerpiece. One hundred μl of Dow Corning No. 555 silicone fluid was introduced to facilitate the studies of molecular weights at the cell bottom (55). It should be emphasized that the extrapolation procedure at the solution silicone interface was rendered difficult by the thickening of the boundary. However, it was found possible to obtain consistent molecular weights by enlarging the pictures 20-fold in the photographic enlarger and consistently choosing the midpoint between the fringes of the solution-silicone boundary as the location of the cell bottom. The concentration of the original sample was determined in arbitrary units in a 12 mm, 2° sector synthetic boundary cell (valve type) at 59,780 rpm. All sedimentation patterns were photographed as described above; the photographs were enlarged 20-fold in a photographic enlarger and traced directly onto 1 mm graph paper. The tracings were

measured in accordance with procedures recommended by Schachmann (52).

The weight average molecular weights at the meniscus and the bottom of the cell were determined from the relations (56):

$$M_m = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{(dc/dx)_m}{x_m c_m}$$

$$M_b = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{(dc/dx)_b}{x_b c_b}$$

where M and $\bar{v}$ are the molecular weight and partial specific volume, respectively; R is the gas constant; T is the absolute temperature; ρ is the density of the protein solution; ω is the angular velocity in radians per second; and dc/dx is the concentration gradient; c_m and c_b represent the concentration of the protein at the cell meniscus and the cell bottom, respectively, and can be calculated from the measurements using the following equations (57):

$$c_m = c_o - \frac{0.1}{F \cdot x_m^2} \sum_{n=0}^{n_x} x_n^2 z_n$$

$$c_b = c_o + \frac{0.1}{F \cdot x_b^2} \sum_{n=x}^{n_b} x_n^2 z_n$$

where c_o is the original concentration (determined in the synthetic boundary cell); 0.1 is the value of the interval between tabulated readings along the x axis; F is the enlargement factor (20 in this particular case); x_m, x_b, and x_n are the

distances of the meniscus, bottom of the cell, and nth interval, respectively, from the axis of rotation; z_n is the ordinate; n_x is the number of intervals needed to bring the ordinate to zero, and n=0 and n_b correspond to the meniscus and cell bottom intervals.

Approximately 0.8 per cent solutions of CHTG-B in 0.1 M NaCl - 0.001 M HCl, pH 3.0, gave the most satisfactory results. To confirm association at higher pH values, one run was performed in sodium phosphate buffer, ionic strength 0.1, pH 7.0.

6. Molecular weight by light scattering

All measurements were performed at a wave length of 436 mμ and at room temperature (23 ±2°C.) with the Brice-Phoenix light scattering photometer (58). A rectangular cell with a capacity of 40 ml was used. Twenty-five ml of an approximately 0.7 per cent solution of CHTG-B in 0.1 M NaCl - 0.001 M HCl, pH 3.0, (the exact concentration was determined by absorbancy measurements at 280 mμ) was clarified by filtration through millipore filters (pore size 0.47 μ) (51), and introduced into the cell. The turbidity of the solution was determined at 90° followed by the addition of subsequent 5 ml portions of solvent previously clarified by the above procedure. After mixing, turbidity measurements were repeated. Three 5-ml additions could be made before the cell had to be emptied. This was done by withdrawing 20 ml of the solution, thereby making room for four additional solvent additions. In this way, a range of concentration between 0.5 and 0.07 per cent could be covered without emptying

of the cell completely at any time. The turbidity of the solvent was determined after thorough rinsing of the cell with millipored solvent and subtracted from the readings of the protein solutions. The data were plotted as Hc/τ against concentration and extrapolated to c=0 to obtain 1/M in accordance with the equation (59):

$$\frac{Hc}{\tau} = \frac{1}{M} + 2\,Bc.$$

The corresponding Debye factor, H, was calculated according to the equation:

$$H = [32\pi^3 n_o^2 (n - n_o)^2/c^2]/3\lambda^4 N$$

where c is the concentrations in g/ml; n is the refractive index of the solution and n_o that of the solvent; λ is the wave length of the incident light in vacuo expressed in cm, and N is Avogadro's number. Substitution of the values for π, N, and λ reduces the equation to

$$H = 15.2 \times 10^{-5} n_o^2 (n - n_o)^2/c^2$$

for wave length 436 mμ. The quantity $(n - n_o/c)$ is a constant (refractive increment) for a given solute-solvent system, and can be determined by means of a Differential Refractometer. Since the refractive increment does not vary significantly from protein to protein (60), a value of 0.1940 at $\lambda = 436$ mμ was assumed. This is also the value found for CHTG-A by Wilcox et al. (19). The refractive index of the solvent (n_o for water)

was assumed to be 1.34. From these values, the Debye factor, H, was calculated to be 1.025×10^{-5}.

B. Results and Discussion

1. Isoelectric point

In Figure 6 the mobilities of CHTG-B are plotted as a function of pH. Positive values of μ correspond to migration of the protein to the cathode, negative values indicate migration to the anode. The zymogen shows zero mobility at pH 5.2 (= Isoelectric Point), in good agreement with data from Keller et al. (2) and Kubacki and co-workers (35), who found 5.1 and 5.2, respectively. It should be emphasized that the value reported herein may not be absolutely correct. It is well known that the mobility of proteins at a given pH is dependent on the buffer system used. In the above experiments, acetate buffer was used on the acidic side of the isoelectric point and cacodylate on the basic side. It is therefore felt that an error of $\pm$0.1 pH units may be possible.

2. Partial specific volume

Table VII summarizes the results obtained by measuring the density of five solutions of CHTG-B in 0.1 M NaCl - 0.001 M HCl, pH 3.0, containing different concentrations of protein. The values calculated for V_{app} were independent of protein concentration and revealed $0.733 = \bar{v}$ as the average figure, a result common to several globular proteins.

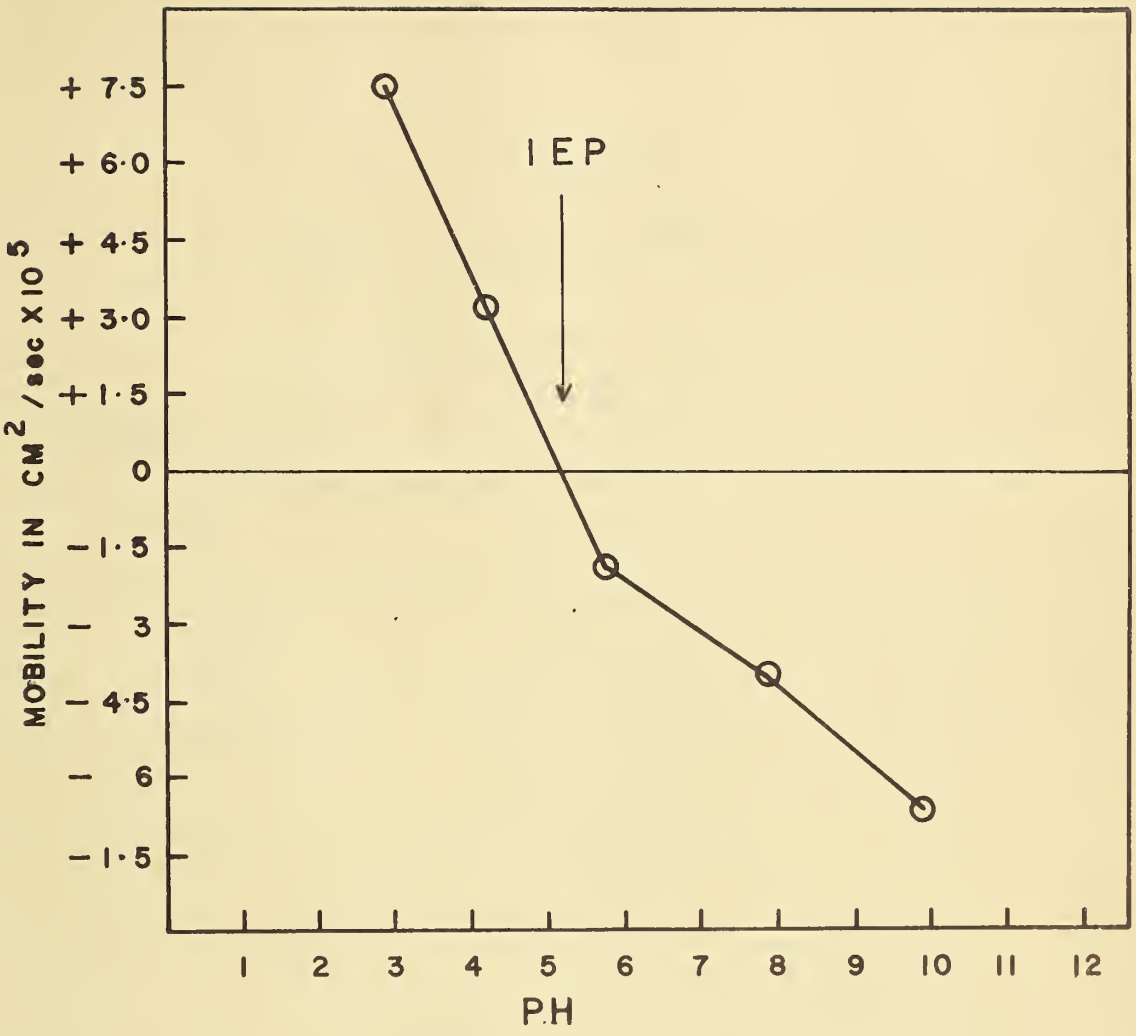


Figure 6

Isoelectric Point (IEP) of CHTG-B

Table VII

Determination of V_{app} from Density Measurements

Concentration of protein solution	Density of solution	V_{app}
(%)	(g/ml)	
1.438	1.00595	0.734
1.258	1.00549	0.733
1.040	1.00489	0.735
0.816	1.00444	0.730
0.644	1.00387	0.731
	Best average value $= \overline{v}$	0.733

3. The sedimentation constant as a function of pH and protein concentration

Figure 7 presents the plot of sedimentation constants as a function of protein concentration at four pH values. It becomes immediately apparent that CHTG-B shows normal concentration dependence at and below pH 4.0. The positive slope at pH 5.0 and above is assumed to be due to association rather than changes in the tertiary structure since the molecular weight of a 0.8 per cent solution of the zymogen at pH 6.98 was found to approach 45,000 (see III.B.5). The $s_{20,w}$ values of the protein in 0.1 M NaCl - 0.001 M HCl, pH 3.0, over the concentration range 0.1 to 2 per cent all fall closely to the same line given by least squares as $s_{20,w} = 2.58\ (\pm 0.02) - 0.105\ (\pm 0.01)\ c$, standard errors being given in parentheses. Drawing of the best straight lines through the available points at each of the other pH values and extrapolation to zero protein concentration yield intrinsic sedimentation constants very close to 2.60 S. These data are in excellent agreement with observations made by Smith et al. (36), who report a value of 2.49 for $s^{\circ}_{20,w}$ at pH 3.88. The latter figure was obtained without making the correction for adiabatic expansion and contraction (61). Application of this correction results in raising $s^{\circ}_{20,w}$ by 2.5 per cent to 2.55 S. Unfortunately, no investigations were carried out by these workers in more alkaline solutions. Keller, Cohen and Neurath, when examining anionic component 2 of bovine pancreatic juice, reported a value of 2.53 S

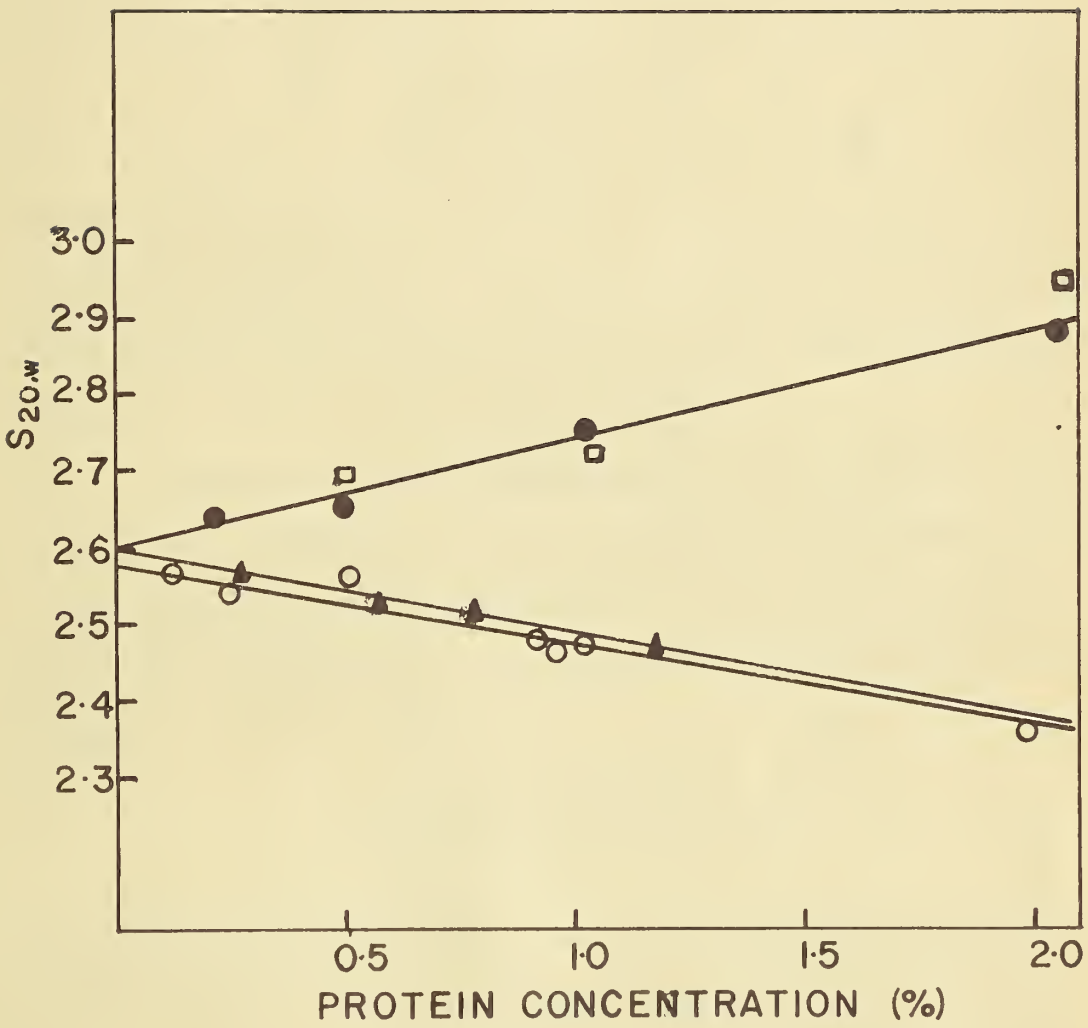


Figure 7

Plot of Sedimentation Constants Versus Protein Concentration for CHTG-B
Open circles - pH 3.0
Triangles - pH 4.0
Closed circles - pH 5.0
Squares - pH 6.98

for $s^{\circ}_{20,w}$. Representative photographs of a sedimentation-velocity run on CHTG-B in 0.1 M NaCl - 0.001 M HCl at pH 3.0 are illustrated in Plate C.

4. Intrinsic viscosity

Figure 8 shows the graph obtained by plotting the reduced viscosity (η_{spec}/c) of CHTG-B versus concentration. Over the concentration range 0.27 to 0.93 per cent zymogen in 0.1 M NaCl - 0.001 M HCl, pH 3.0, the points fall close to a straight line given by $\eta_{spec}/c = 2.63\ (\pm 0.02) + 0.699\ (\pm 0.08)\,c$. This value is considerably lower than any found in the literature and demonstrates the highly symmetrical and compact structure of the CHTG-B molecule previously noted by Smith (36) when studying the frictional coefficient of the zymogen.

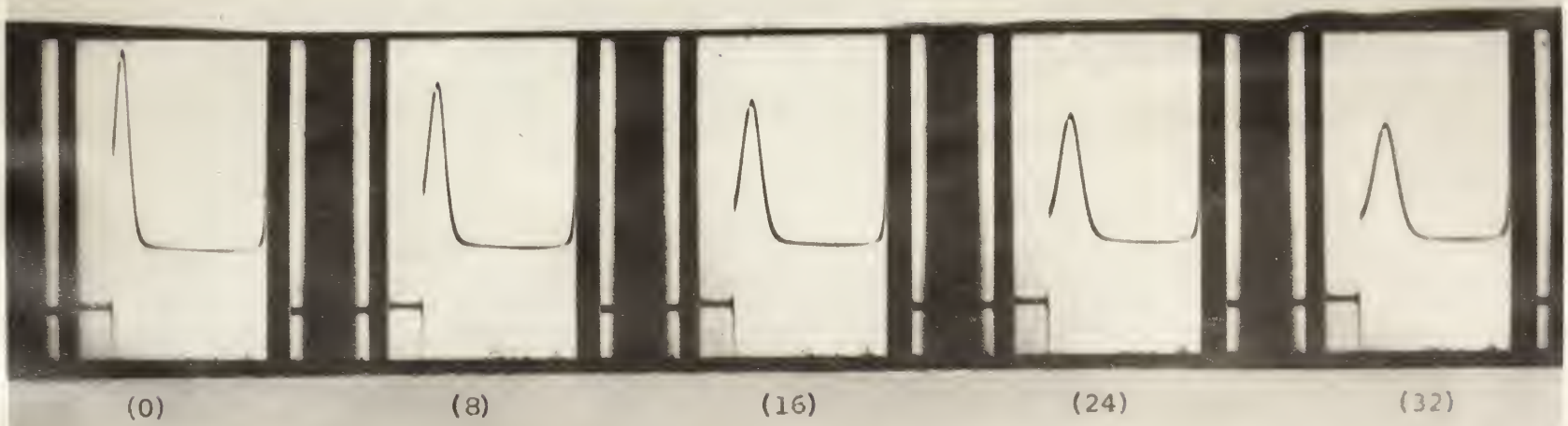
5. The molecular weight of chymotrypsinogen-B

a. Calculation from sedimentation-viscosity data

An estimate of the molecular weight was obtained by combining the sedimentation and viscosity data through the Scheraga-Mandelkern equation (62):

$$M = \left[\frac{N \cdot s \cdot \eta^{1/3} \cdot \eta_o}{\beta\,(1 - \bar{v}\rho)} \right]^{3/2},$$

where N is Avogadro's number; s is the intrinsic sedimentation constant of the protein; $[\eta]$ the intrinsic viscosity; η_o the viscosity of water at 20°C.; $\bar{v}$ the partial specific volume of the protein; and ρ the density of water at 20°C. The parameter β depends on the hydrodynamic characteristics of the solvated protein. Bearing in mind the low intrinsic viscosity of CHTG-B,



Sedimentation velocity run of CHTG-B in 0.1 M NaCl - 0.001M HCl, pH 3.0. Speed 59,780 r.p.m.; bar angle 50°; protein concentration, 1%; time after reaching full speed (minutes) indicated under each photograph.

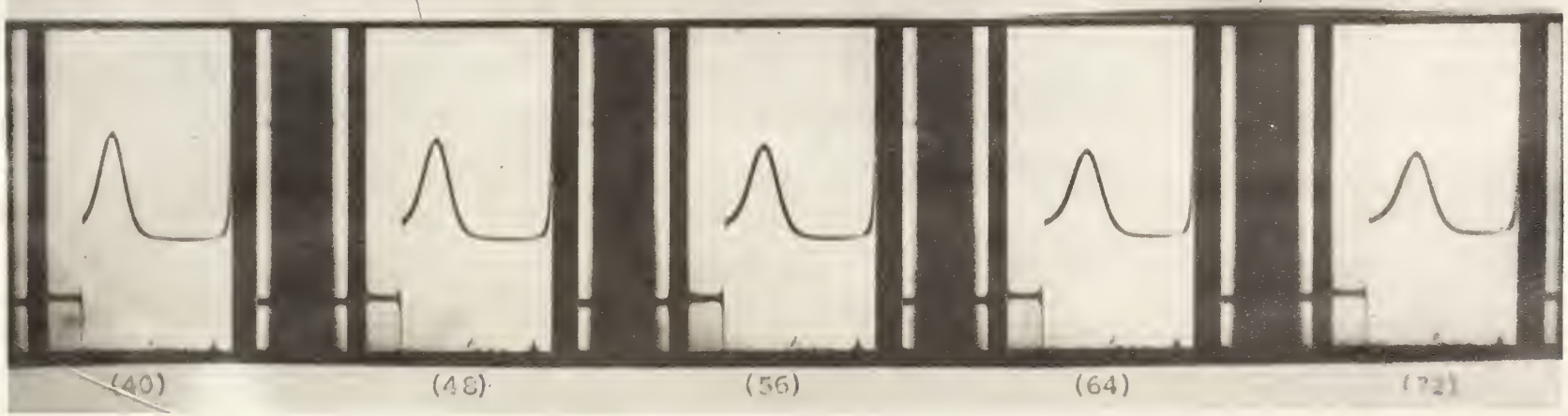


Plate C

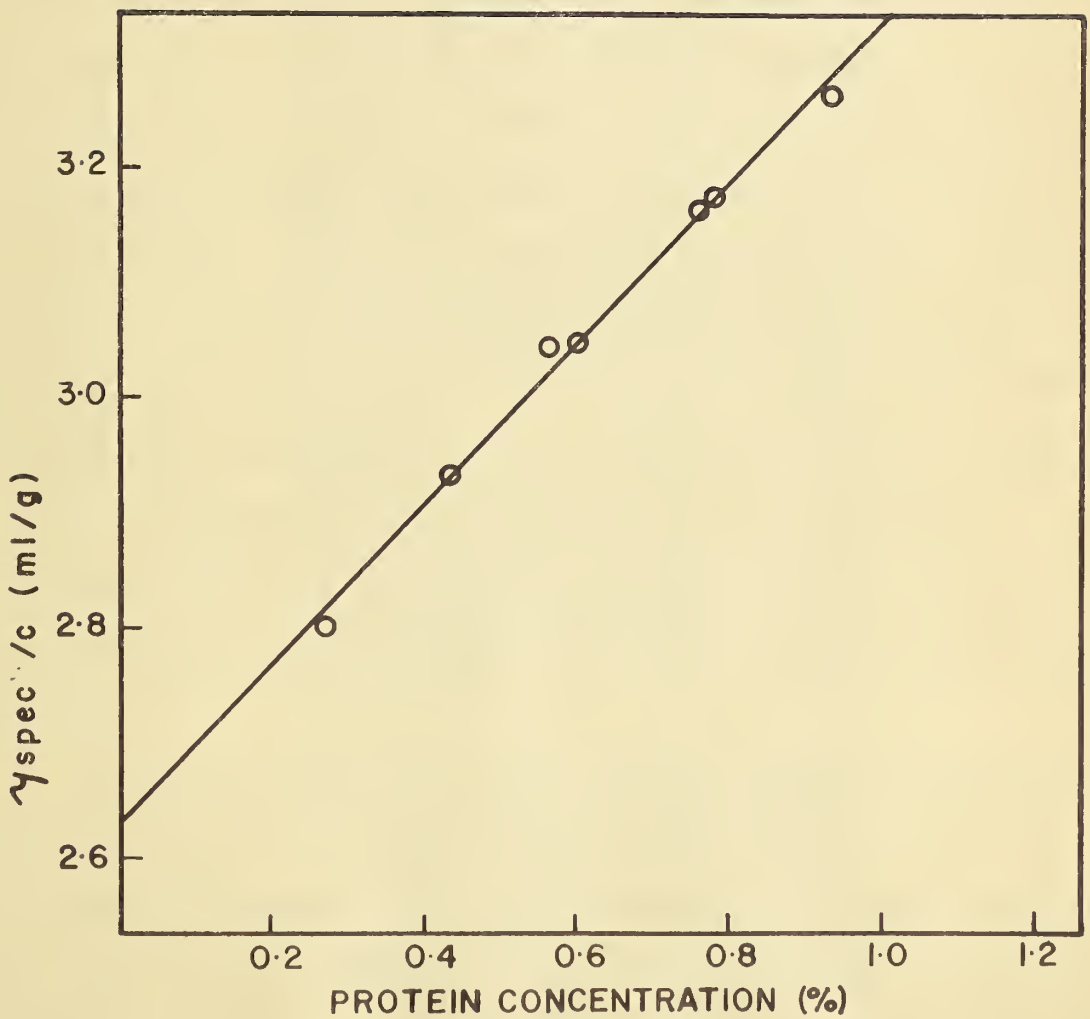


Figure 8

Plot of Reduced Viscosity Versus Protein Concentration for CHTG-B in 0.1M NaCl-0.001M HCl, pH 3.0

a value of 2.12×10^6 for β seems reasonable (the value for a rigid sphere). The accuracy of the method is 5 to 10 per cent. Combining the values for the sedimentation constant, the intrinsic viscosity, and the partial specific volume yields a molecular weight of 24,000 ±2,000.

b. Approach to sedimentation equilibrium

A typical schlieren photograph of the approach to sedimentation equilibrium and the corresponding run in the synthetic boundary cell for CHTG-B are shown in Plate D. Table VIII lists the molecular weights obtained at the meniscus and cell bottom of runs performed with different preparations of the zymogen. Considering figures calculated at the meniscus of the cell only, the molecular weight was found to be 25,400 ±300. It can be seen from the standard errors that calculations done at the cell bottom are less accurate. This stems from the fact that the extrapolation procedure at the solution-silicone interface was rendered difficult by the thickening of the boundary. Combination of both sets of results yields a mean molecular weight of 24,700 ±900, the standard error being within acceptable limits.

To confirm the fact that CHTG-B exhibits association rather than a shape change in solutions more basic than pH 4.0, an approach to sedimentation equilibrium was carried out on a 0.8 per cent solution of the zymogen in 0.1 M sodium phosphate buffer, pH 6.98. The centrifuge was operated at 8,766 rpm. Calculations revealed a molecular weight of 45,200 at the

Archibald run on 0.8% CHTG-B in 0.1M NaCl - 0.001M HCl, pH 3.0. Speed 12,590 r.p.m.; bar angle 75°; temperature 20°. Times after full speed are (A) 0 minutes, (B) 16 minutes, (C) 32 minutes, (D) 48 minutes, (E) 64 minutes. Photograph (F) is a synthetic boundary run for the same solution and condition.

Plate D

Table VIII

Molecular Weight of CHTG-B from Approach to Sedimentation-Equilibrium. Solution of Protein (0.8%) in 0.1 M NaCl-0.001 M HCl, pH 3.0.

Preparation	Time of picture	Mol. wt. at meniscus	Mol. wt. at cell bottom
	(min.)		
CHTG-B-C	16	25,779	23,169
	32	25,002	23,581
CHTG-B-ASF	16	25,430	23,153
	32	25,859	25,119
	48	25,737	25,119
CHTG-B-G	16	24,595	23,417
	32	25,547	24,659
Average value		25,421±303	23,897±714
Best value		24,700±900	

meniscus and 43,500 at the bottom of the cell. These figures indicate the presence of 90 per cent dimers and demonstrate that the positive slope of the s versus c plot at pH 6.98 is indeed due to association.

c. Light scattering

The values of Hc/τ, as obtained for CHTG-B at pH 3.0, are plotted as a function of protein concentration in Figure 9. The best straight line drawn by the method of least squares intercepts the ordinate at 4.0×10^{-5} being the reciprocal of the weight average molecular weight. It was calculated to be 25,000 $\pm$400. The value for the interaction constant B equals 0.051.

d. Summary

The molecular weight results obtained in this laboratory are summarized in Table IX. Good agreement has been demonstrated for the different methods employed and may be interpreted as strong evidence for the homogeneity of the preparations used. The values calculated from light scattering, approach to sedimentation equilibrium, and sedimentation-viscosity data converge toward 24,500 as the most probable molecular weight of CHTG-B. Smith *et al*. arrived at a molecular weight of 22,200 (corrected from 21,600 in order to take into account the adiabatic cooling of the rotor) and 24,300 (corrected from 23,600) by sedimentation diffusion measurements for CHTG-B and CHT-B, respectively (36). Kassel and Laskowski (29) obtained a minimum molecular weight of 23,086 from a complete amino acid analysis. Considering

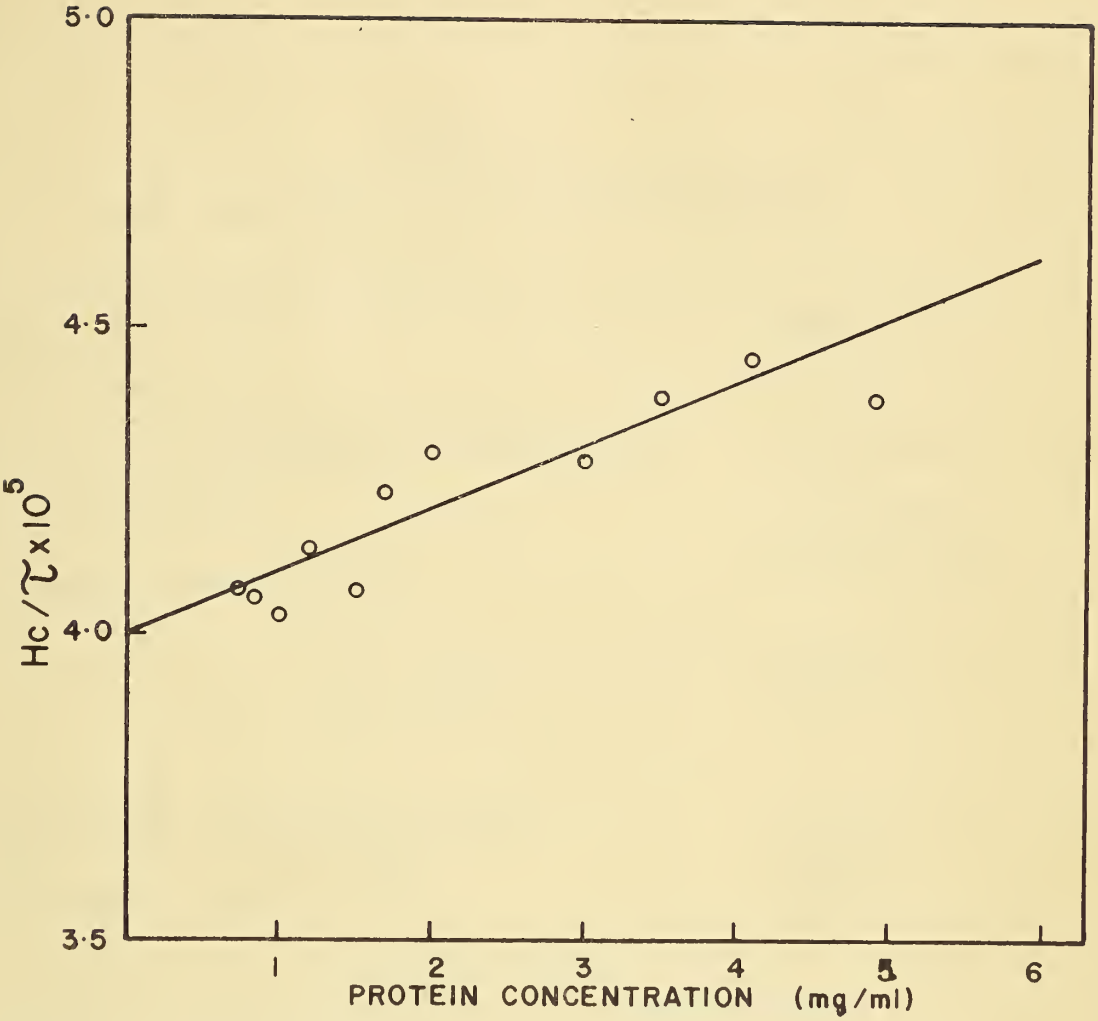


Figure 9

Plot of $\frac{Hc}{\tau}$ versus Protein Concentration for CHTG-B in 0.1M NaCl-0.001M HCl, pH 3.0

Table IX

Molecular Weight of Chymotrypsinogen-B

Method	Mol. wt.
Approach to sedimentation equilibrium	24,700 ±900
Light scattering	25,000 ±400
Sedimentation-viscosity	24,000 ±2000
Best average value	24,500
Sedimentation-diffusion (37)*	22,200
Amino acid analysis (29)	23,086

*Numbers in parentheses refer to Bibliography.

the entirely different approach to the purification of the zymogen in Laskowski's laboratory, the results are in reasonable agreement.

C. Conclusions

Table X presents a comparison of physico-chemical parameters of CHTG-A and CHTG-B. It demonstrates great similarity between the two zymogens with regard to both molecular size and shape. Considering the low value of intrinsic viscosity of CHTG-B together with a somewhat smaller frictional coefficient, it may be speculated that the latter protein molecule is even more compact and symmetrical than CHTG-A in solution.

Table X

Physico-chemical Constants of CHTG-B and CHTG-A

Parameter	CHTG-B	CHTG-A
$s^{\circ}_{20,w}$	2.55 S (36)*	2.54 S (62)
	2.58 S,[a] (pH 3 and 4)	2.49 S (36) (pH 3.0)
	2.60 S,[a] (pH > 4)	2.58 S (19) (pH 7.5)
$\bar{v}$	0.733[a]	0.721 (63)
$[\eta]$	2.63 ml/g[a]	3.26 ml/g[c]
f/f_o	1.11 (36)	1.17 (63)
	1.14[b]	
$D^{\circ}_{20,w}$	10.3×10^{-7} cm^2/sec (36)	9.009×10^{-7} cm^2/sec (19) (pH 3)
		9.48×10^{-7} cm^2/sec (19) (pH 7.5)
mol. wt.	24,500[a]	25,100 (19)
Isoelectric point	5.2[a]	9.5 (64)
	5.2 (35)	9.1 (35)
	5.1 (2)	

*Numbers in parentheses refer to Bibliography.
[a]Data taken from this study.
[b]Calculated from $s^{\circ}_{20,w}$ and mol. wt. according to Svedberg and Pederson (54, page 40).
[c]Calculated from the value of f/f_o by the relationship of Scheraga and Mandelkern (62).

IV. SPECIFICITY OF CHYMOTRYPSIN-B

As pointed out in the Introduction, the specificity of CHT-B has received little attention. Apart from qualitative studies on the hydrolysis of synthetic substrates (25), nothing is known concerning its action on proteins or polypeptides. Taking into consideration the wide application of proteolytic enzymes to studies of the primary structures of proteins, it seemed of interest to investigate the specificity of this enzyme with regard to a more complex molecule and compare it to the well-established specificity of CHT-A_4. Glucagon was chosen as a substrate for two reasons: first, its primary structure has been elucidated by Bromer, Sinn and Behrens (44) using chymotrypsin-A_4 among other proteinases; second, it contains a variety of amino acids and may be obtained commercially in a homogeneous form.

A. Methods

1. Digestion of substrates with CHT-B and CHT-A_4

a. Digestion of glucagon

Crystalline glucagon,* reference standard (Lot No. 258-234 B-167-1) and crystalline porcine glucagon* (Lot No. 258-234

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*Both preparations were kindly donated by Eli Lilly Laboratories, Indiana.

B-214 CP) were used during this investigation. The conditions of digestion were similar to those of Bromer, Sinn, and Behrens (44), although in some experiments the ratio of enzyme to substrate was different. Chymotrypsinogen-B was activated as described in the Appendix. After activation, STI was added (weight ratio of trypsin to STI was 1 to 5) in order to reduce the free tryptic activity to a minimum. CHT-A_4, three times recrystallized, was purchased from Worthington Biochemical Corporation, Freehold, N. J. All digestions were carried out at pH 8.0 and 37 $\pm$0.2°C. with a Jacobsen-Leonis autotitrator (65), manufactured by Ole Dich, Instrument Maker, Copenhagen, Denmark, in conjunction with a titrator type TTT-1 from the Radiometer Corporation, Copenhagen, Denmark. The assembly was used with a temperature controlled reaction vessel designed for small volumes (66). In a typical experiment, 8.5 mg (2.44 μmoles) of glucagon in 10 ml of demineralized water were introduced into the reaction vessel. The pH of the suspension (glucagon is sparingly soluble at pH 8.0) was brought to 8.0 and 0.5 mg of CHT-A_4 or CHT-B (molar ratio enzyme to substrate 1 to 120) was added. The pH was kept at 8.0 by the automatic addition of 0.1 N NaOH (carbonate free). After two hours of digestion, a 5 ml aliquot was withdrawn from the digestion mixture, brought to pH 2.5 by the addition of 1 M HCl, heated in a boiling water bath for 3 minutes to inactivate the enzyme, and freeze-dried. To the remaining solution, 0.25 mg of enzyme was added and the digestion was continued for a further two

hours. The reaction was then stopped as above and the solution was freeze-dried. Sometimes eight hours of digestion were allowed to simulate the conditions of Bromer et al. (44).

b. Digestion of peptides and amino acid derivatives

With the exception of benzoyl-L-leucine ethyl ester (BLEE) all synthetic substrates were purchased from Mann Research Laboratories, Inc., New York 6, N. Y. Benzoyl-L-leucine ethyl ester was prepared according to the method described by Ottesen and Spector (67) for the preparation of benzoyl-L-tyrosine ethyl ester. In the above procedure, slow addition of water to the ethanolic solution of BLEE did not result in a crystalline precipitate (as was reported for the tyrosine ester) but rather in an oil which was collected and redissolved in 95 per cent ethanol. It was possible to obtain amorphous BLEE by pouring the ethanolic solution of the oil into a large volume of water. The resulting precipitate was collected by filtration, washed with water on the filter, and dried in a vacuum desiccator over P_2O_5 (m.p. 99 - 101°C.; over-all yield 31%). Due to its limited solubility in water, BLEE was dissolved in 95 per cent ethanol (660 mg in 10 ml) and 100 µl of this solution was added to 2.4 ml of 0.01 M Tris buffer, pH 8.0, containing 0.02 M $CaCl_2$ and 0.1 M KCl for assays. N-acetyl-tyrosine ethyl ester, ATryEE, and L-phenylalanine ethyl ester were dissolved in the same buffer and digested in the pH stat as described for ATEE in the Appendix. The digestions of L-leucine ethyl ester, glycyl-L-leucyl-glycine, and L-leucyl-L-leucyl-L-leucine could not be

followed in the pH stat. Solutions of the latter substrates (10 mM) were digested with CHT-B and CHT-A_4 (molar ratio of enzyme to substrate 1 to 500) at 23 $\pm$2°C. for 12 hours. Aliquots of the digests were examined by high-voltage electrophoresis at pH 2.2 as described in the following paragraph.

2. Separation of peptides and amino acids

The products of digestion were isolated by a procedure essentially similar to the "finger print" technique of Katz et al. (68). The freeze-dried digests were dissolved in 1 ml of demineralized water and 100 or 200 µl (0.244 µmoles or 0.488 µmoles) aliquots were applied as round spots (1.5 cm in diameter), 3 cm apart, 15 cm from the anode of a 54 cm x 54 cm Whatman No. 3 paper. After wetting with pH 3.7 buffer (pyridine (1) - acetic acid (10) - water (289)) the sheet was subjected to electrophoresis (40 V per cm) for 2-1/2 or 3 hours. The paper was dried at room temperature under a fume hood and cut into 3 cm wide strips which contained the partially separated peptides. The strips were sewn to sheets of the above size and run for 16 hours in the descending butanol - acetic acid - water (4:1:5) chromatographic system. The completed "finger prints" were dried at room temperature and sprayed very lightly with ninhydrin solution (0.05% ninhydrin in 2 N acetic acid - absolute ethanol (1:3) (69)). The colors were allowed to develop overnight at room temperature. Some peptides containing tryptophane and valine did not stain when subjected to the above treatment. They could be detected under ultraviolet light.

All spots were cut out and eluted with 5 to 10 ml of demineralized water. The samples were dried *in vacuo*, taken up in 0.75 ml of constant boiling HCl, and hydrolyzed in sealed, evacuated tubes for 16 hours at 110 ±2°C. After removal of the hydrochloric acid *in vacuo*, the residue was taken up in a minimum amount of demineralized water and subjected to two dimensional electrophoresis and chromatography according to the method of Richmond and Hartley (70). This procedure differs from the one described in the last paragraph in two points. The buffer employed for high-voltage electrophoresis consists of 4 per cent formic acid and 0.4 per cent pyridine in water, pH 2.2, and the solvent system for paper chromatography is made up from butanol - butyl acetate - acetic acid - water (19:1:5:25). Standard amino acid mixtures (0.1 μmole of each amino acid) were always subjected to hydrolysis, electrophoresis, and chromatography along with the peptides to allow accurate identification and quantitation.

3. Quantitation of amino acids

The method employed was essentially that of Hanes and coworkers (69). The amino acids were detected and identified by spraying the paper with detection spray and heating to 65°C. for 10 minutes. The resulting spots were cut out together with blanks of similar size and wetted twice with borate buffer, pH 11.3, to remove traces of ammonia. After drying with a stream of hot air in a wind tunnel the papers were cut into 5 mm squares and introduced into 15 ml test tubes, calibrated at

10 ml. The tubes were flushed with a stream of high purity nitrogen for 10 minutes and 2.0 ml of ninhydrin (0.38% ninhydrin, 0.06% hydrindantin in methylcellosolve, - 1 M acetate buffer, pH 5.5) was added to each tube. The color was developed by heating the tube in boiling water for 20 minutes. The volume of each tube was made up to 10 ml by the addition of 50 per cent ethanol and the optical density of each solution was read at 570 mμ. The optical density readings of the blanks (generally between 0.15 and 0.20) were subtracted from those of the amino acids, and the corrected values were translated into μmoles of amino acids. The method was found to yield an accuracy of ±15 per cent.

4. N-terminal amino acids of digests

The reaction of peptide mixtures with fluorodinitrobenzene (FDNB) was conducted according to Sanger and Thompson (71). Suitable aliquots of the digests (0.1 ml) were taken to dryness and dissolved in 500 μl of 1 per cent trimethylamine. To this was added a solution of 50 μl of FDNB in ethanol. After standing for 2 hours, a few drops of water and trimethylamine solution were added and the excess FDNB was extracted with three 5-ml portions of ether. The aqueous solution was taken to dryness and the residue was dissolved in 0.5 ml of constant boiling HCl and hydrolyzed in a sealed, evacuated tube at 110°C. for 12 to 16 hours. All the following operations were conducted in the dark to minimize destruction of the DNP-amino acids by light. The hydrolyzate was diluted to 6 volumes with

water and extracted with four portions (4 ml) of ether. The etheral and aqueous solutions were evaporated to dryness. Standard mixtures of DNP-amino acids (Mann Research Laboratories, Inc.) were subjected to the same treatment to allow the estimation of recoveries.

The ether soluble DNP-amino acids were separated by the tert-amyl alcohol - phthalate system of Blackburn and Lowther (72). Sheets of Whatman No. 4 paper (54 cm x 54 cm) were buffered with pH 6.0 phthalate buffer (0.05 M) and dried at room temperature. The residues of the hydrolyzates were taken up in a minimum amount of reagent grade acetone and applied to the paper as small round spots (0.8 cm in diameter). Irrigation of the papers was affected with tert-amyl alcohol saturated with the buffer; water, saturated with tert-amyl alcohol was present at the bottom of the chromatography tank. The descending procedure was found to yield satisfactory results. The water soluble DNP-amino acids were subjected to descending chromatography in the butanol-acetic acid-water (4:1:5) system (73). After separation and identification, the spots containing the DNP-amino acids together with blanks of the same size were cut out and immersed in 4 ml of 1 per cent sodium bicarbonate in 15 ml tubes. The color was eluted by placing the tubes in a water bath at 55° to 60°C. for 15 minutes and estimated at 360 mμ.

B. Results and Discussion

During the studies of the amino acid sequence of glucagon, Bromer et al. (44) demonstrated that CHT-A_4 catalyzed the hydrolysis of 5 peptide bonds adjacent to aromatic amino acid residues (2 tyrosine, 2 phenylalanine, and 1 tryptophane residue) along the polypeptide chain. The separation of 6 peptides was achieved by chromatography on Dowex-X2 resin and each peptide was characterized with regard to its amino acid composition and partial sequence. It was further shown that no two peptides formed overlapping sequences, which meant that the proteolysis of glucagon by CHT-A_4 was essentially complete after 8 hours of hydrolysis (80-90% as revealed by N-terminal analysis of digests). Additional studies on digests of the hormone with trypsin and subtilisin enabled these investigators to propose the amino acid sequence of glucagon. It was therefore possible for the author to determine the bond(s) hydrolyzed by the enzyme from amino acid analyses of the peptides separated by the two-dimensional electrophoresis and chromatography system.

1. Digestion of glucagon with CHT-A_4 and CHT-B.

a. Evidence from peptide maps

Glucagon was digested with CHT-A_4, CHT-B (chromatographed on CM-cellulose to remove trypsin), and CHTG-B activated with trypsin (to which STI had been added after activation to inhibit trypsin). The resulting peptide maps are shown in Figures 10 and 11. Each circle represents a peptide, and the numbers in the circles refer to Table XI which lists the amino acid sequence

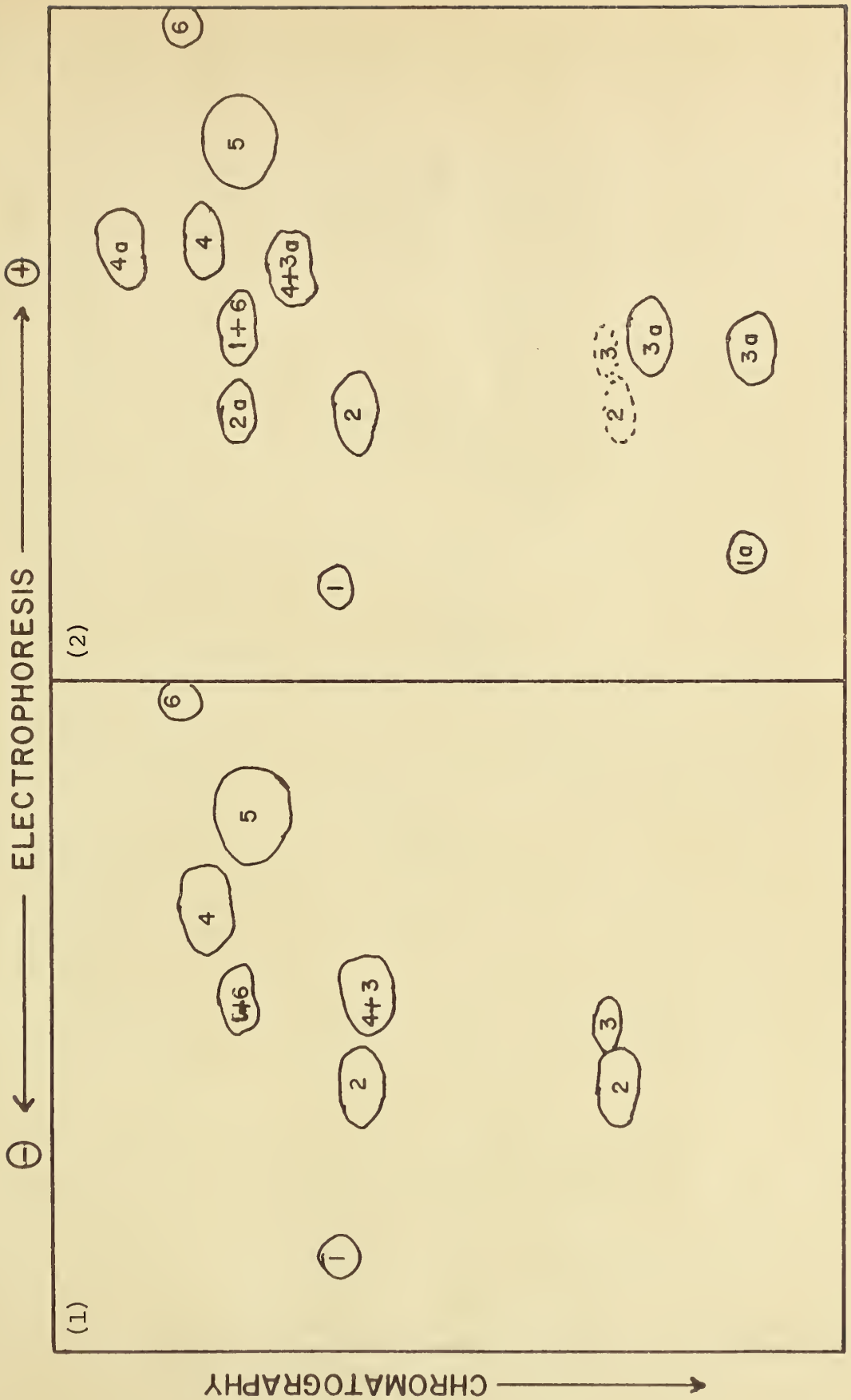

Figure 10

Peptide Maps of 4 hr. Digests of Glucagon (for explanation, see text). (1) Digest with CHT-A_4; (2) Digest with CHT-B. Broken circles indicate smaller quantities.

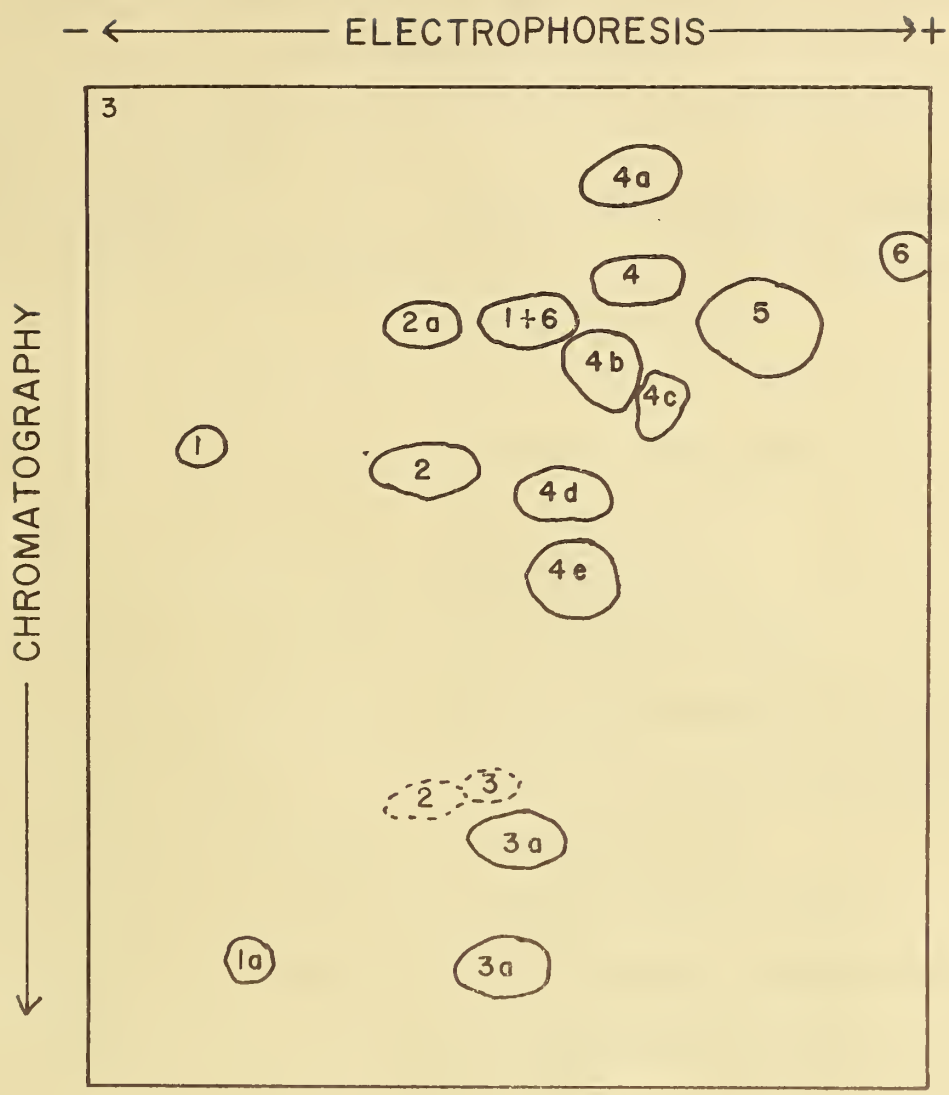

Figure 11

Peptide Map of 4 hr. Digest of Glucagon with CHT-B (STI added to inhibit trypsin) (for explanation, see text)

Table XI

Amino Acid Sequences of Peptides Liberated from Glucagon by CHT-A_4 and CHT-B

No. of peptide in Figures 10,11	Amino acid sequence of peptide
1	thr-ser-asp-tyr
1a	leu
2	leu-met-aspNH_2-thr
2a	met-aspNH_2-thr
3	val-gluNH_2-try
3a	val-gluNH_2-try-leu
4	leu-asp-ser-arg-arg-ala-gluNH_2-asp-phe
4a	asp-ser-arg-arg-ala-gluNH_2-asp-phe
4b	leu-asp-ser-arg
4c	arg-ala-gluNH_2-asp-phe
4d	arg-ala-gluNH_2-asp-phe-val-gluNH_2-try
4e	arg-ala-gluNH_2-asp-phe-val-gluNH_2-try-leu
5	his-ser-gluNH_2-gly-thr-phe
6	ser-lys-tyr
1 + 6	thr-ser-asp-tyr-ser-lys-tyr
4 + 3	leu-asp-ser-arg-arg-ala-gluNH_2-asp-phe-val-gluNH_2-try
4 + 3a	leu-asp-ser-arg-arg-ala-gluNH_2-asp-phe-val-gluNH_2-try-leu

of each peptide. In cases where a peptide bond was only partially hydrolyzed, peptides were produced corresponding to a larger fragment of the glucagon molecule. These peptides are designated by a composite number to indicate that their total amino acid composition corresponds to the sum of two smaller peptides found on the peptide map. Figure 12 relates the findings of the peptide maps to the primary structure of glucagon.

Before presenting a detailed discussion of the various digests, a few observations of general application will be described. It will be noted that at least two spots on each peptide map bear identical numbers. For example, both spots with number 2 were found to contain the amino acids leucine, methionine, aspartic acid, and threonine. It is believed that partial deamidation of the asparagine residue present in glucagon at this position is responsible for the encountered observation. Since both components show identical mobilities during high-voltage electrophoresis, deamidation must have occurred just prior to or during chromatography. By the same token, the circles numbered 3a supposedly result from partial deamidation of a glutamine residue. When digests of glucagon with CHT-B were first examined, the peptide val-glu NH_2-try (spot 3) could not be found as a ninhydrin positive spot. However, due to the presence of tryptophane, ultraviolet light proved to be more efficient. A reexamination of some sprayed papers after two to three weeks revealed that very faint blue

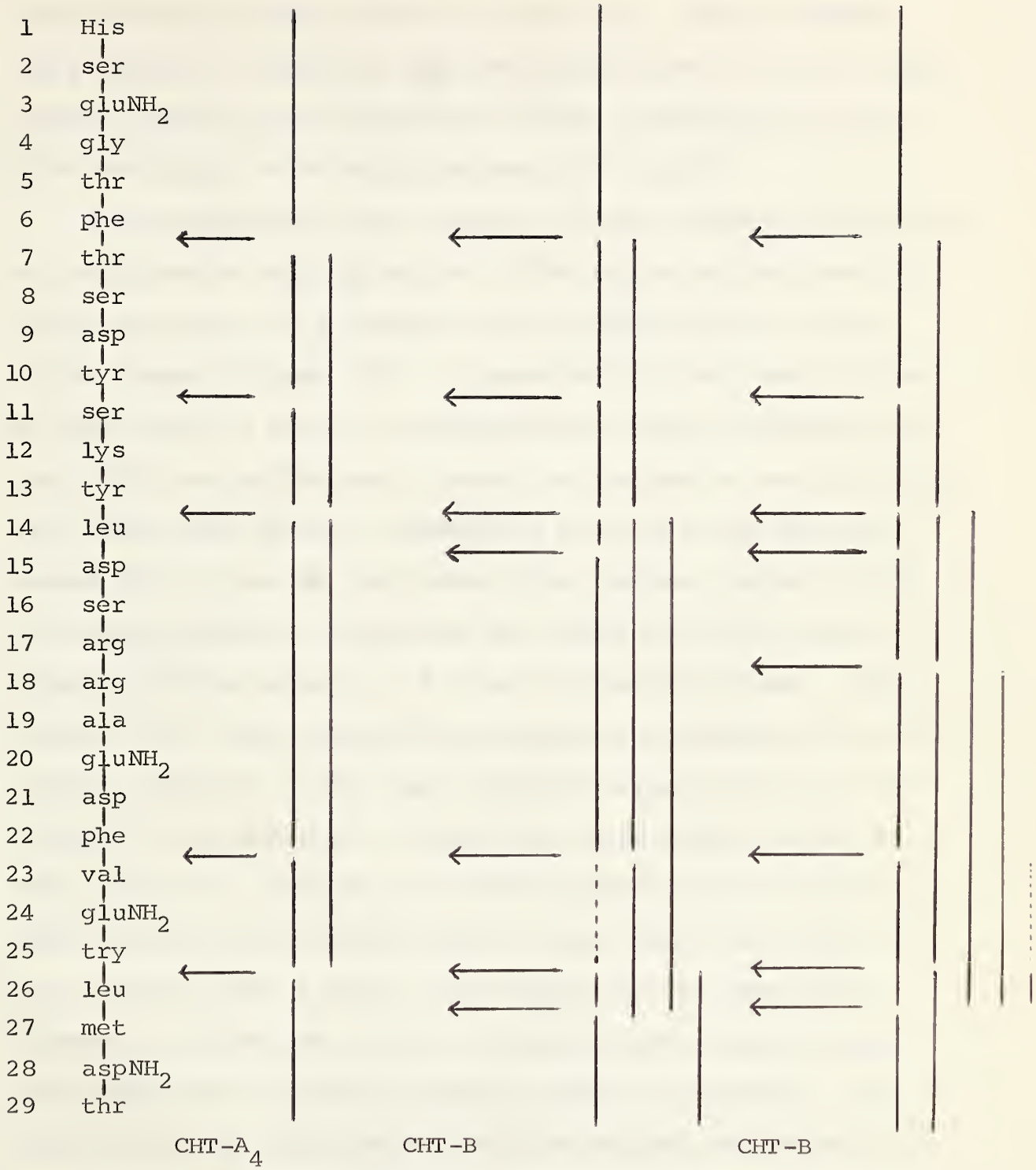

Figure 12

The action of CHT-A_4, CHT-B (trypsin free) and CHT-B (STI added to inhibit trypsin) on glucagon. The lines represent the digestion products isolated from the various peptide maps.

spots had developed where ultraviolet light had demonstrated the presence of these peptides (3 and 3a). These findings can probably be explained by observations made in other laboratories, where it was found that valine containing peptides gave low color yields with ninhydrin (44,74,75).

Base consumption as a function of time during the hydrolysis of glucagon with CHT-A_4 and CHT-B (trypsin-free) gave the first indication of a lesser degree of specificity of the latter enzyme (Figure 13). A comparison of the total uptake of NaOH during a period of digestion of 4 hours demonstrates that CHT-B has hydrolyzed a larger percentage of available peptide bonds than CHT-A_4. Assuming a pK of 7.3 for the amino groups (76) it can be calculated from the base uptake during the first 2 hours of digestion that CHT-A_4 and CHT-B have cleaved the equivalents of 5.7 and 7.4 peptide bonds, respectively. Two other interesting features are apparent from this figure. Firstly, a very fast reaction during the first five minutes of digestion is followed by a much slower uptake of NaOH. Secondly, when an additional aliquot of the enzyme is added to the digestion mixture two hours after the start of the reaction, only a small and rapid uptake of NaOH can be observed, followed by a rate of reaction even smaller than that immediately before the second addition of enzyme. The implications of these results will be further discussed in a later paragraph of this section. Figure 10 presents peptide maps of digests of glucagon with CHT-A_4 and CHT-B (free of

trypsin) which confirm the assumption made on the basis of Figure 13. Peptide map 1 (glucagon-CHT-A_4) essentially corroborates the results of Bromer et al. (44). The only difference between the two sets of data is found in the presence of peptides 4 + 3 and 1 + 6 which results from the incomplete cleavage of one tyrosine and one phenylalanine bond. Even 8 hours of digestion did not change the results significantly. However, in the work of Bromer et al., it is possible that strong adsorption to the Dowex-X2 resin may have prevented the detection of these larger fragments. Apart from this discrepancy, the results fully agreed with the well-established specificity of CHT-A_4 and served as a comparative basis throughout this study.

Peptide map 2 in Figure 10 and Table XI demonstrate that CHT-B not only cleaves bonds also split by CHT-A_4 but in addition catalyzes the hydrolysis of peptide bonds adjacent to leucine residues in glucagon. This becomes evident from the appearance of 5 peptides not present in digests of glucagon with CHT-A_4. Further evidence thereof was found during the early stages of this study when it was observed that trypsin used for the activation was not completely inhibited by STI and thus caused the cleavage of the arg-arg bond of glucagon (positions 17 and 18). Figure 11 shows a representative peptide map of such a digest. Identification of the amino acids of each peptide made possible only one interpretation of the results. Tryptic digestion causes the cleavage of the arg-arg

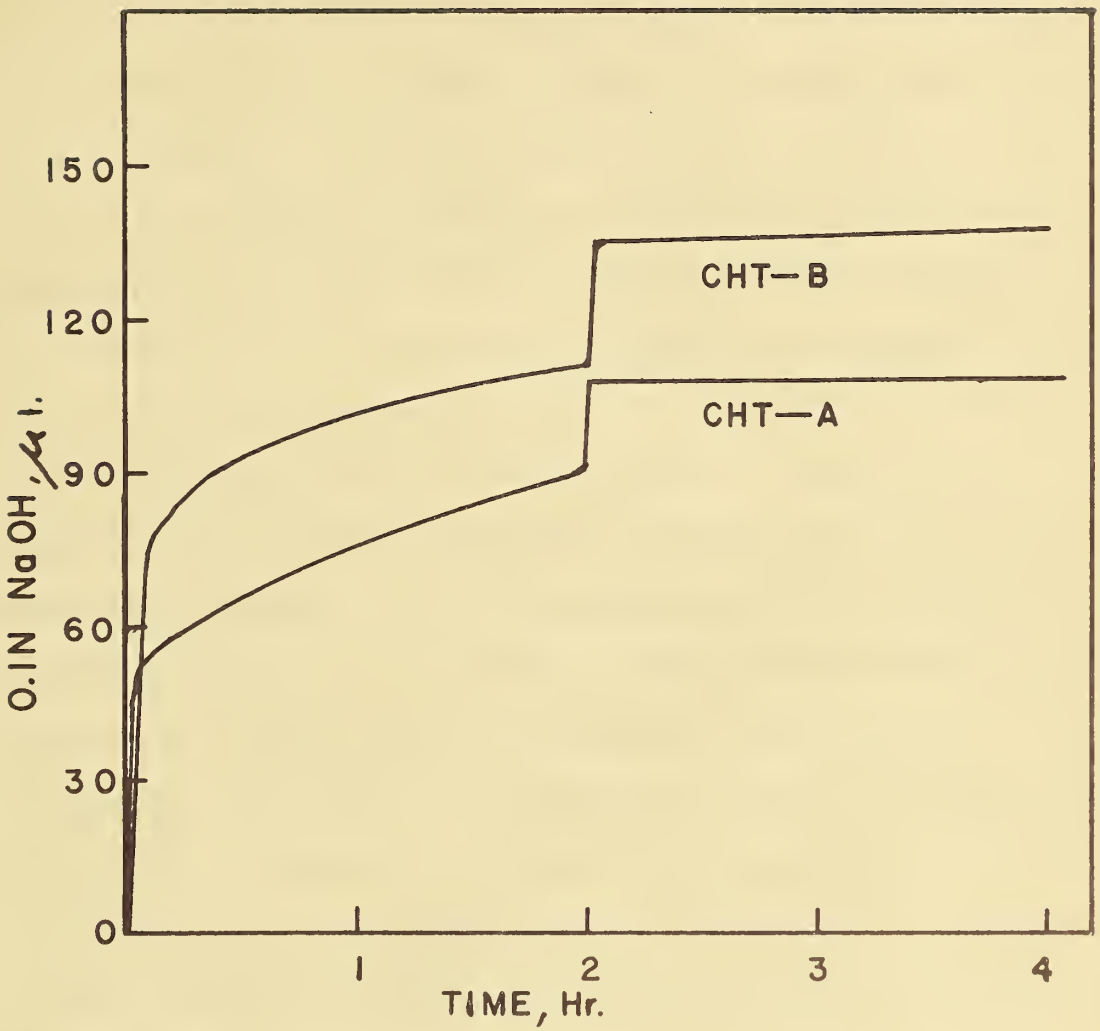

Figure 13

Digestion of Glucagon by CHT-A_4 and CHT-B (Trypsin Free) Followed by Recording pH Stat; 2.44 µmoles of Glucagon in 10.0 ml, 500 µg enzyme; pH 8.0, T=35°C.

bond of peptides 4, 4a and 4 + 3a. These results gain more weight in the light of the findings of Bromer, Sinn and Behrens (44) which demonstrate that this particular bond in glucagon is highly susceptible to tryptic hydrolysis.

Table XII presents semi-quantitative results of the estimation of some peptides liberated by CHT-A_4 and CHT-B (4 hour digestion). Between 40 and 50 per cent of the major part of the glucagon sequence is recovered as amino acids after elution and hydrolysis. Clearly, these results do not represent absolute quantities of peptides liberated, since the recoveries of peptides during elution from the paper cannot be measured easily. A quantitative comparison of the same peptide liberated from different digests will be of some significance, however. With this point in mind, evaluation of the data summarized in Table XII demonstrates some significant differences between the two digests. For example, 0.40 μmoles of peptide 2 per μmole of glucagon are found in digests with CHT-A_4; this quantity is reduced to 0.29 μmoles in digests with CHT-B, since part of peptide 2 is cleaved adjacent to the leucine residue and gives rise to 0.18 μmoles of peptide 2a plus leucine.

It should also be mentioned that CHT-B was found to catalyze an even larger number of peptide bonds when incubated with glucagon under different conditions (0.2% glucagon, ratio of enzyme to substrate 1:50, 37°C., 4 hrs.). Although no detailed studies of representative peptide maps have been

Table XII

Quantities of Peptides Present in Digests of

1 μmole of Glucagon by CHT-A_4 and CHT-B

Number of peptide	CHT-A_4 (μmoles)	CHT-B (μmoles)
1	0.08	0.12
1a	-	0.32
2	0.40	0.29
2a	-	0.18
4	0.12	0.27
4a	-	0.15
5	0.44	0.42
6	0.09	0.09
1 + 6	0.33	0.30
4 + 3	0.27	-

undertaken, some available evidence indicates that bonds adjacent to aspartic acid are also cleaved.

From the above data it is impossible to make accurate statements about the rates at which peptide bonds relative to one another are hydrolyzed. However, results from digests terminated after 5 minutes (end of rapid reaction) indicate that both enzymes attack 1 phenylalanine bond (position 6 along the polypeptide chain) and 1 tyrosine bond (position 13) before any other. These findings are in accord with the results obtained from 4 hour digests of glucagon with CHT-B. Figure 13 indicates that only the bonds adjacent to amino acid residues 6 and 13 are hydrolyzed to the extent of 100 per cent. CHT-A_4 in addition cleaves the peptide bond between residues 25 and 26 (involving the carboxyl group of tryptophane) completely.

b. Evidence from N-terminal amino acids

One possible way of confirming the above results was to react aliquots of the digests with FDNB and separate the formed DNP-amino acids after hydrolysis. The splitting of leucine bonds in glucagon should make the demonstration of N-terminal aspartic acid and methionine possible. Moreover, results from such studies were expected to give a better quantitative picture of the peptides present in the digests.

Table XIII shows the results from 2 and 4 hour digests of glucagon with CHT-A_4 and CHT-B. The liberation of 0.01 μmole of aspartic acid from digests with CHT-A_4 does not seem significant and probably stems from an impurity of the enzyme itself.

Table XIII

DNP-Amino Acids of Peptides Present in Digests of Glucagon by CHT-A_4 and CHT-B (μmoles per μmole of Glucagon)

DNP-amino acid	CHT-A_4 digestion		CHT-B digestion	
	2 hours	4 hours	2 hours	4 hours
Asp	0.01	0.01	0.25	0.31
Met	-	-	0.36	0.45
Val	0.49	0.56	0.32	0.38
Thr	0.68	0.75	0.59	0.68
Ser	0.18	0.20	0.19	0.23
Leu	1.25	1.40	0.73	0.79

Together with the absence of DNP-methionine, it demonstrates unequivocally that CHT-A_4 attacks peptide bonds in glucagon adjacent to aromatic amino acid residues only. The findings with respect to CHT-B, however, can be explained only on the basis of hydrolysis of two leucine peptide bonds in addition to those involving aromatic residues. Another interesting point is also apparent from this table. Although neither enzyme has achieved the complete hydrolysis of all susceptible peptide bonds of glucagon after 2 hours of digestion, no significant increase in the quantities of N-terminal amino acids is found after 4 hours. Denaturation of the enzyme as a possible explanation for this phenomenon can be ruled out, because an additional aliquot of CHT-A_4 or CHT-B was added after 2 hours.

2. Digestion of synthetic substrates with CHT-A_4 and CHT-B

Data of the hydrolysis of some synthetic amino acid esters and peptides by CHT-A_4 and CHT-B are presented in Table XIV. It is obvious that no essential difference between the two enzymes is found with regard to the substrates tested. Both catalyze the hydrolysis of leucine esters and neither has any demonstrable activity against such peptides as L-trileucine or glycyl-L-leucyl -glycine.

In a recent communication, Konigsberg and Hill (77) summarized part of their findings in the following sentences: "One of the most useful observations made in this study was

Table XIV

Hydrolysis of Amino Acid Esters and Peptides by CHT-A_4 and CHT-B

Substrate	CHT-A_4	CHT-B
	(%)	(%)
ATEE	100[a]	100
ATryEE	37	22
L-phenylalanine ethyl ester	6	2
BLEE	1.6	1.2
L-leucine ethyl ester	complete hydrolysis in 12 hours	
L-leucyl-L-leucyl-L-leucine	- - no hydrolysis - -[b]	
Glycyl-L-leucyl--glycine	- - no hydrolysis - -	

[a]The value of k' for the hydrolysis of ATEE represents 100%.

[b]Time of hydrolysis, 12 hours.

the finding that chymotrypsin (A_4) or pepsin can hydrolyze a particular peptide linkage at a much faster rate than other potentially sensitive residues. . . The possibility that a longer sequence may determine the ability for a particular bond to be cleaved, as suggested by Hirs, Moore, and Stein* or the possibility that the conformation of the peptide itself may govern the differential rates of hydrolysis is open to future investigation and could provide additional information leading to a more refined concept of enzyme action." There is sufficient evidence from the present study that these suggestions apply equally well to the proteolytic enzyme CHT-B. Studies on the uptake of NaOH as a function of time (reaction rate) together with data from N-terminal analyses, indicate quite clearly that doubling of the enzyme-substrate ratio after some period of digestion does not produce very significant changes. Furthermore, it is known that CHT-A_4 hydrolyzes peptide bonds adjacent to leucine residues of the B-chain of insulin (79), of sheep pituitary adrenocorticotrophic hormone (80), of bovine ribonuclease (81), and others, but not of glucagon. Finally, CHT-B cleaves leucine peptide bonds in glucagon but not in simple peptides as shown above. Whereas the latter two observations may well result from structural features of the substrates (e.g. neighboring groups), the decline of the reaction rate over the period of time studied could also arise

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*Hirs, Moore and Stein (78).

from competitive inhibition of the enzyme by the products. No evidence for or against this interpretation can be cited from the presented work and it remains to be seen whether this speculation will be proved or disproved by further studies.

C. Conclusions

The hydrolysis of glucagon by CHT-A_4 results in the liberation of eight different peptides, six of which have been demonstrated by Bromer et al. (44) before. Only peptide bonds adjacent to aromatic amino acid residues are cleaved, although some at a faster rate than others. Even after 8 hours of digestion, the reaction is not completed.

CHT-B, in addition to the above peptide bonds, also catalyzes the cleavage of linkages adjacent to leucine residues in glucagon. Since studies on the purity of the enzyme preparation did not reveal the presence of a separate leucine esterase as a contaminant, it is concluded that this activity is an inherent property of CHT-B. Its availability from beef pancreas glands in a highly purified form makes the enzyme an attractive tool for studies of the primary structures of proteins.

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APPENDIX

1. Determination of proteolytic activity

a. Activation of chymotrypsinogen-B

Chymotrypsinogen-B (1% solution in 0.1 M Tris, pH 8.0) when incubated with trypsin (Worthington Biochemical Corporation) at 0°C. was found to attain maximal activity in less than 10 minutes (molar ratio of trypsin to chymotrypsinogen-B 1:40). In a typical experiment, 12 mg of CHTG-B was dissolved in 1.2 ml of 0.1 M Tris buffer, pH 8.0. One hundred µl of this solution was diluted 100-fold in order to determine the absorbancy at 280 mµ. Another 100 µl was introduced to the reaction vessel of the pH stat for the determination of free chymotryptic activity. To the remainder, 25 µl of a 1 per cent solution of trypsin in 0.001 M HCl was added at zero time. After 10 minutes of incubation at 0°C., 50 µl of the activation mixture was added to 9.95 ml of 0.005 M HCl to terminate the reaction. Twenty-five µl of the dilution (containing approximately 0.0005 mg of enzyme nitrogen) was used for assays in the pH stat.

b. Determination of proteolytic activity in the pH stat

The apparatus used was identical to that described in section IV.A.1. A 10 mmolar solution of N-acetyl-L-tyrosine-ethyl ester in 0.01 M Tris, containing 0.02 M $CaCl_2$ and 0.1 M KCl, was used for both chymotrypsins. For the determination of tryptic activity benzoyl-L-arginine-ethyl ester (10 mmolar)

was employed. In all experiments, 2.5 ml of substrate was introduced to the reaction vessel. The pH was adjusted to 8.0 by the addition of 0.1 M NaOH (carbonate free), and suitable aliquots of enzyme were added. Under the conditions used, the reaction followed zero order kinetics for at least 15 minutes. The slope of the plot of uptake of NaOH versus time was determined and a value of the k'_{m2} was calculated according to the formula:

k'_{m2}=meq of substrate hydrolyzed per min. per mg of enzyme N. Activities of the enzymes against ATryEE, L-phenylalanine-ethyl ester, and BLEE were measured accordingly.

2. Determination of deoxyribonucleolytic activity

The assay employed in this laboratory essentially corresponds to Kurnick's colorimetric methyl green assay (50). Solutions of CHTG-B eluted from CM-cellulose columns by citrate buffers were dialyzed overnight against 0.1 M sodium acetate buffer, pH 5.0, in order to remove the citrate ions (citrate ions are known to inhibit DNase I). Freeze-dried preparations were dissolved in demineralized water and diluted suitably the substrate (30 mg of deoxyribonucleic acid-methyl green) (Worthington Biochemical Corporation) was dissolved in 0.05 M Tris buffer (150 ml), 0.0075 M with respect to magnesium sulfate, pH 7.5, and equilibrated in a water bath kept at 37° $\pm$0.5°C. for a few hours. At zero time, 1 ml of the diluted enzyme was added to 15 ml of substrate solution at 37°C. At one-minute intervals,

2.0 ml aliquots were removed and added to 3 ml of inhibiting solution (1 part of 0.33 M sodium citrate and 3 parts of 0.05 M Tris, pH 7.5). The tubes were allowed to stand at least 12 hours at room temperature. The absorbancy at 640 mμ was determined, using a water blank. The initial reaction rate per minute, ΔA/min, was obtained by plotting absorbancy readings versus time. The specific deoxyribonucleolytic activity of each solution was evaluated using the formula:

$$\text{Units /mg} = \frac{\Delta A/\text{min} \times 2.5 \times 1{,}000}{99{,}500 \times \text{mg protein/ml of digestion mixture}}$$

where 99,500 is the molar extinction coefficient of bound methyl green as determined by Kurnick; the concentration of protein in mg/ml was obtained by multiplying the absorbancy of the protein solution at 280 mμ by a factor of 0.90. Standard DNase I with a specific activity of 1.0 unit/mg, supplied by Worthington Chemical Corporation, with the substrate, was assayed accordingly, and the results were corrected to account for differences in the various samples of substrate used.

3. Details of electrophoresis experiments

Plate A:

picture (a): CHTG-B (0.8%) in glycine-HCl buffer, ionic strength 0.1, pH 2.96; voltage 150; current 12 mAmp.; time of pictures, ascending boundary 112 min., descending boundary 67 min.

picture (b): CHTG-B (0.8%) in acetate buffer, ionic strength 0.1, pH 4.51; voltage 175; current 12mAmp.; time of pictures, ascending limb 169 min., descending limb 171 min.

picture (c): CHTG-B (0.8%) in cacodylate buffer, ionic strength 0.1, pH 6.04; voltage 167; current 12 mAmp.; time of pictures, ascending limb 84 min., descending limb 230 min.

Plate B:

picture (d): CHTG-B (0.8%) in Tris-HCl buffer, ionic strength 0.1, pH 7.5; voltage 160; current 12 mAmp.; time of pictures, ascending limb 72 min., descending limb 69 min.

picture (e): CHTG-B (0.8%) in glycine-NaOH, ionic strength 0.1, pH 9.48; voltage 163; current 10.5 mAmp.; time of pictures, ascending limb 66 min., descending limb 67 min.

picture (f): CHTG-B (0.8%) in acetate buffer, ionic strength 0.1, pH approximately 4.5 (for exact details, see Section II.B.5); voltage 180; current 11.5 mAmp.; time of pictures, ascending limb 95 min., descending limb 98 min.

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